Role of the RpoE sigma factor and two-component Cpx systems in biofilm formation of *Salmonella enterica* subspecies *enterica* serovar Enteritidis

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By

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ABSTRACT

The cell envelope of the Gram negative bacteria is a complex multilayered structure that functions to protect bacteria from various stressors. The cell envelope is involved in maintenance of cellular shape and rigidity, transport of nutrients and waste, cell division as well as in growth and metabolism. Thus, it is crucial for bacteria to ensure the integrity of the cell envelope in response to environmental assaults and maintain a continuous, semi-permeable barrier provided by the cell envelope. Bacteria have evolved specialized response systems, called extracytoplasmic stress responses systems (ESRs), that function to sense the damage of the cell envelope and accordingly regulate the genes essential to combat the stress, thus restoring envelope and intracellular homeostasis. Two of these ESR systems, the RpoE sigma factor and the Cpx two-component system, are involved in regulating genes in response to the envelope stresses. These regulatory systems have been linked to pathogenesis and biofilm formation in several Gram negative pathogens, although the complex network mediated by these systems, along with their interaction, remain unclear. Accordingly, a first goal of this thesis was to investigate the individual and synergistic role of the RpoE and Cpx (CpxA and CpxR) regulatory systems in their abilities to affect biofilm formation in *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis). For this purpose, I generated in-frame deleted Δ*rpoE*, Δ*cpxR* and double-deleted Δ*rpoE*/Δ*cpxR* strains. These mutant strains were assessed for underlining characteristic differences in biofilm formation compared with wild-type (WT) *S.* Enteritidis. It was determined that *cpxR* (a response regulator of Cpx system) and *rpoE/cpxR* knock-out mutant strains of *S.* Enteritidis resulted in decreased biomass in biofilms compared to that of the parental WT strain. The cells in the *cpxR* knock-out mutant biofilm additionally exhibited an unusual filamentous phenotype. In contrast, the *rpoE* knock-out mutant strains exhibited architecture and the cellular morphology similar to WT, which formed benchmark *Salmonella* biofilms with decreasing porosity and increased biomass overtime. Based on the above results on biofilm formation, I report that RpoE by itself did not significantly influence the biofilm formation in *S.* Enteritidis. However, in conjunction with CpxR, the RpoE sigma factor might co-regulate essential genes required for biofilm formation indicating an integrated role of these two ESRs in biofilm formation. The above findings revealed that CpxR individually, as well as in combination with RpoE sigma factor, played an important role in biofilm formation of *S.* Enteritidis. Accordingly, the second goal of this thesis was to characterize the expression of genes under control of the Cpx regulon in *S.* Enteritidis in

response to biofilm formation. For this purpose, comparative transcriptome profiling between WT and *cpxR* mutant strains were performed to identify CpxR-regulated genes involved in biofilm formation. My results suggest that CpxR is involved in regulation of a number of regulatory pathways linked to adhesion, motility, O-antigen biosynthesis and virulence. Some of the operons that were transcriptionally-repressed by deletion of *cpxR* includes, the *peg* and *saf* operon involved in adherence; the *rfb* and *gtr* operon involved in O-antigen synthesis; and the *hsd* operon involved in virulence. Together these results suggest that Cpx controls regulatory pathways affecting adhesion, motility and O-antigen biosynthesis which, together or individually, could impact biofilm formation by *S.* Enteritidis.

Overall, my work has revealed an essential role of CpxR regulatory protein in biofilm formation and highlighted several genes that are directly regulated by CpxR. It offers new insights on the CpxR-regulon required for successful surface adaptation, and helps to elucidate the complex stress-response metabolic pathway utilized by *S.* Enteritidis. To my knowledge, this is the first study providing a transcriptomic analysis of the Cpx regulon in *Salmonella*.

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

-Marie Curie

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1. GENERAL INTRODUCTION

In Gram negative bacteria, the cell envelope is compartmentalized principally into three layers, the outer membrane (OM), the periplasm or the periplasmic space, and the cytoplasmic or inner membrane (IM) (Silhavy *et al.,* 2010). The cell envelope protects the bacteria from an unpredictable and often hostile external environment, but it allows the selective passage of nutrients from outside and waste from inside (Huang *et al.,* 2008). Thus, the cell envelope represents the protective shield for the bacteria and acts as a "first line of recognition and defence" against various environmental threats. Bacteria have evolved sophisticated stress-sensing response systems called as extracytoplasmic stress response (ESRs) systems that can sense the stress on its envelope and alter the gene expression to ease the stress, thus protecting the interface between the bacteria cell and its environment (Rowley *et al.,* 2006; Grabowicz & Silhavy, 2017). The five extracytoplasmic stress response systems characterized in *Salmonella* spp. are Psp (Phage shock protein), Rcs (Regulator of capsule synthesis), Bae (Bacterial adaptive response), Cpx (Conjugative plasmid expression) and RpoE (RNA polymerase sigma factor E σ^E) (Macritchie & Raivio, 2009; Rowley *et al.,* 2006). In my thesis, I will be discussing the current state of knowledge on the Cpx and RpoE stress-signalling systems focussing on their role(s) in biofilm formation which presently is poorly understood.

The RpoE response is potentially activated by the misfolding of proteins in the outer membrane, leading to the release of RpoE sigma factor from the membrane-bound anti-sigma factor, RseA (Ades, 2008). The released RpoE sigma factor binds to the core of RNA polymerase holoenzyme and directs the RNA polymerase to RpoE-dependent promoters, thus assisting the RpoE-dependent gene expression (Rhodius *et al.,* 2006). The second envelope stress response governed by the Cpx is a two-component system controlled by CpxA (sensor kinase) and CpxR (response regulator) (DiGiuseppe & Silhavy, 2003). The Cpx system is thought to be activated by the misfolding of the periplasmic proteins, thus responding to the perturbations in the IM of the cell envelope (Raivio, 2014). Both these systems have linkages with pathogenesis and biofilm formation (see Review by Rowley *et al.,* 2006; Macritchie & Raivio, 2009). However,

understanding how these systems work individually or in tandem to regulate gene expression during pathogenesis and biofilm formation is an ongoing challenge, particularly for non-typhoidal *Salmonella*.

Non-typhoidal *Salmonella* (NTS) are the leading cause of foodborne gastroenteritis worldwide (Majowicz *et al.,* 2010). The public health importance of these zoonotic pathogens is underscored by the fact that from the years 2000 to 2008, infections by NTS serovars accounted for approximately 1.2 million laboratory-confirmed illnesses, 23,000 hospitalizations, and 450 deaths each year in United States alone (Ao *et al.,* 2015). Among NTS, *Salmonella enterica* subspecies *enterica* serovar Enteritidis (hereafter referred to as *S*. Enteritidis) is the most important serovar and owes its great public health importance to an unusually broad host range. This zoonotic pathogen is capable of infecting numerous mammalian, reptile and bird hosts (Bäumler *et al.*, 1998; Suar *et al.,* 2006), consequently creating a large animal reservoir. In addition to a large natural reservoir, *S*. Enteritidis has the ability to form robust biofilms on various materials under different conditions (Korber *et al.,* 1997; Dantas *et al.,* 2018) further enhancing its persistence in the environment. Biofilm-forming ability has been associated with high virulence and oral invasiveness (Kuboniwa *et al.*, 2013; Naves *et al.*, 2008). The switch from the planktonic to biofilm lifestyle in bacteria is mediated by various regulatory systems (Bordi & de Bentzmann, 2011). A large number of these regulatory systems involves extracytoplasmic stress response regulators. Targeting these extracytoplasmic stress response regulators might disfavor the biofilm formation in *S*. Enteritidis. However, the protective umbrella provided by these ESRs and their role in biofilm formation, is yet to be elucidated.

1.1. Study rationale

Biofilms are recognized as the major virulence factor for chronic infections and have serious implications in industrial, environmental and public health sector (Satpathy *et al.*, 2016). Biofilms formed by *Salmonella* are especially widespread in poultry meat processing environments and plays an essential role in survival of *Salmonella* in poultry farms and chicken slaughterhouse, thus contributing to cross-contamination and foodborne infections (Merino *et al.*, 2017). This has a significant economic impact due to medical costs associated with salmonellosis, food spoilages, food recalls, loss of consumer confidence and equipment damage through corrosion. Thus, efforts are made to understand the role of various regulators that contribute to biofilm formation.

Accordingly, the impacts of these two extracytoplasmic stress signal response systems, RpoE and Cpx, were determined in *Salmonella* biofilm formation. These experiments are described in the third chapter of my thesis (Chapter 3). Furthermore, comparative transcriptomics was used to determine the genes regulated during biofilm formation between the WT strain and the *cpxR* mutant strain in Chapter 4. Finally, Chapter 5 summarizes the findings from both the studies and provides a general discussion along with conclusions and future directions. The overall goal of this thesis was to examine the individual and combined roles of the ESR systems (RpoE and Cpx) in biofilm formation and further to identify critical genes associated with the biofilm formation via the Cpx system.

1.2. Hypotheses

1. Significant differential effects will be observed on the growth, motility and auto-aggregation ability of *S.* Enteritidis due to the single deletion of *rpoE* and *cpxR* and double-deletion of *rpoE/cpxR* in *S.* Enteritidis.
2. RpoE and Cpx stress-signalling systems individually and synergistically will have a varying impact on biofilm formation by *S.* Enteritidis.
3. Characterization of Cpx regulon will highlight the number of regulatory pathways that are involved in biofilm formation and are differentially regulated by Cpx system.

1.3. Technical Objectives

- Investigate the role of RpoE and Cpx system in *S.* Enteritidis biofilm formation:
    a. To differentiate the *rpoE*, *cpxR* and *rpoE/cpxR* knock-out mutant strains in terms of their growth, motility and auto-aggregation, essential characteristics for biofilm formation.
    b. To examine whether the deletion of *rpoE*, *cpxR* or both *rpoE/cpxR* causes deficient biofilm formation in *S.* Enteritidis.
- Investigate the interconnected regulatory circuits between Cpx and other regulatory pathways involved in biofilm formation:
    a. To determine whether Cpx system regulates the expression of genes involved in biofilm formation using transcriptomic approach.

b. To access whether any significant change in gene expression is observed between the planktonic and biofilm lifestyle of *S.* Enteritidis regulated by Cpx system.

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streptomycin-pretreated mouse model. Infect Immun.**74:** 632–644.

2. LITERATURE REVIEW

2.1. The Gram negative cell envelope

The Gram negative cell envelope makes the first point of contact with the environment and thus bacteria have evolved a complex, multilayered envelope for their protection. The Gram negative cell envelope is composed of three layers; the outer membrane (OM), the periplasmic space and the cytoplasmic or the inner membrane (IM) (Costerton *et al.,* 1974). The text below provides a brief description on the organization, composition, and the functions of the above described membranes. Figure 2.1.1 provides a schematic representation of the Gram negative cell envelope.

2.1.1. The Outer Membrane

The outer membrane is the outermost layer of the cell envelope which is specific to Gram negative bacteria and is absent in Gram positive bacteria (Silhavy *et al.,* 2010). The outer leaflet of the outer membrane is composed of lipopolysaccharides (LPS); whereas, the inner leaflet is made up of phospholipid (Kamio & Nikaido, 1976). LPS is generally considered to be an endotoxin and forms an essential component of the bacterial cell comprising lipid A, core oligosaccharide and highly-variable O-antigen polysaccharide (O-antigen) (Shimada *et al.,* 2012). The O-antigen is made of repeating unit of polysaccharide and is an important component of the outer membrane for the bacterial survival. Human pathogens like *Salmonella enterica* and *Burkholderia cepacia* uses O-antigen to avoid phagocytosis (Murray *et al.,* 2003; Murray *et al.,* 2006; Saldías *et al.,* 2009). Notably, the O-antigen, especially the genes involved in the biosynthesis of the O-antigen, are targeted for vaccine development. In *Shigella*, one of the strategy for vaccine development includes covalently linked O-antigen to carrier proteins and use as a parenteral conjugate vaccine. (Levine *et al.,* 2007). The outer membrane consists of two classes of proteins, lipoproteins and β-barrel proteins. Lipoproteins are composed of lipid moieties whereas β-barrel proteins also referred

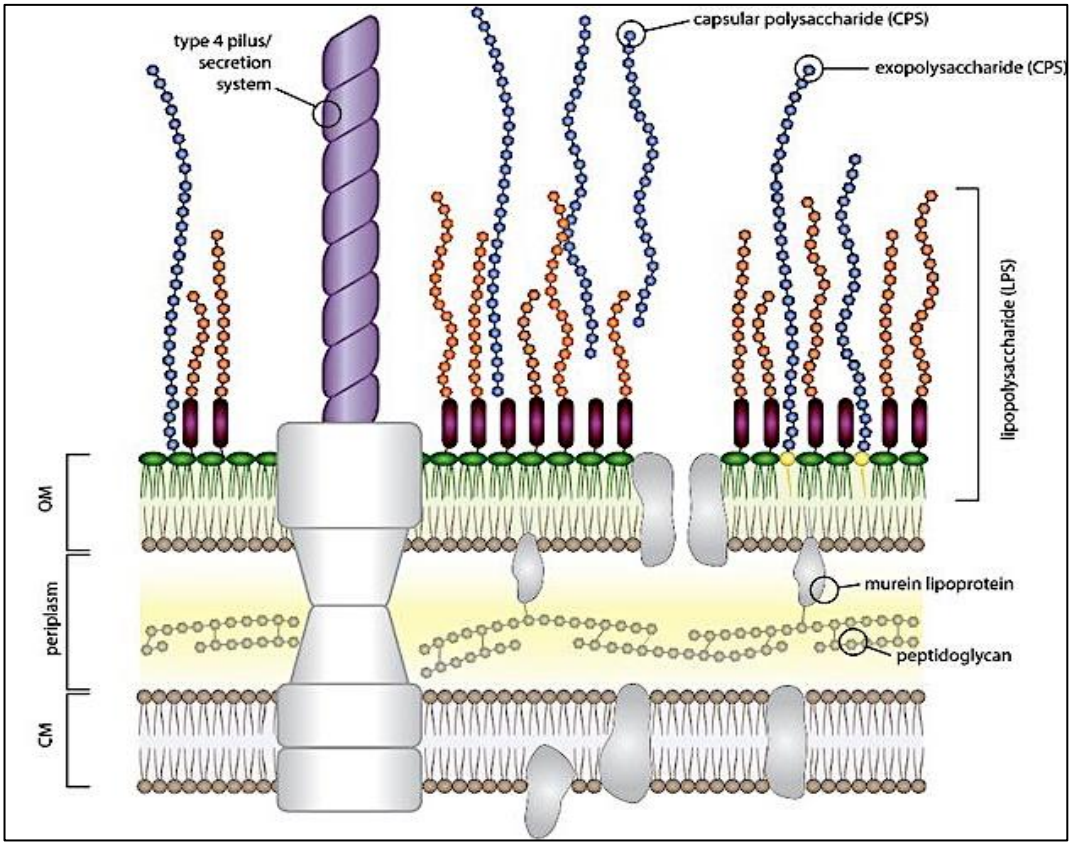

Figure 2. 1. Schematic representation of the Gram negative bacterial cell wall. The periplasm spanning between the outer membrane and the cytoplasmic membrane or inner membrane. The peptidoglycan layer is depicted as grey in the periplasm. The outer leaflet of the outer membrane are lipopolysaccharides depicted in green. The cell surface protein (described below) such as pili or needle-like secretion system are represented in the image above. Adapted from Filloux & Whitfield (2016).

to as porins are β-sheet wrapped into cylinders (Sankaran & Wu, 1994). The outer membrane performs a variety of different functions including enzymatic activity, substrate-specific transporters, diffusion porins and signal transduction [review by Silhavy *et al.* (2010)].

2.1.1. The Periplasm

The periplasm is a densely packed aqueous layer located between the IM and OM, containing soluble proteins, solutes and the peptidoglycan cell wall. The peptidoglycan cell wall is made up of repeating units of N-acetyl muramic acid (NAM) and N- acetyl glucosamine (NAG) (Vollmer *et al.,* 2008).Due to its rigidity, peptidoglycan essentially gives shape to the bacteria [see Yang *et al.,* 2016]. Without the peptidoglycan or disruption in the synthesis of peptidoglycan can result in the cells losing its characteristic shape (Typas *et al.,* 2017). Besides peptidoglycan, the periplasm includes various proteins such as hydrolytic enzymes involved in breakdown and transport of nutrients, protein-folding chaperones involved in envelope biogenesis and solute-binding transport proteins involved in chemotaxis and sugar and amino-acid transport (Thomas *et al.,* 2010).

2.1.2. The Inner Membrane

The inner membrane is a phospholipid bilayer consisting of three main categories of IM proteins. These include integral IM proteins, lipoproteins and peripheral membrane proteins. The varied functions performed by IM proteins include lipid biosynthesis, protein secretion, protein translocation and energy production (Dalbey *et al.,* 2011).

2.1.3. Cell Surface Structures

The bacterial cell surface includes essential components that are synthesized to perform various roles involved in biofilm formation and pathogenicity. Many of these surface structures are specifically attached to the OM, but some span the entire cell envelope (i.e., cellulose, flagella, fimbriae and secretion systems). Flagella play an important role in chemotaxis and motility, and usually are up to ~20 μm in length (Wiedemann *et al.,* 2015). In biofilm formation, flagella are required for the bacteria to reversibly attach to the surface. Fimbriae are fine, hair-like protein appendages which are required for the adhesion (Yaron & Römling, 2014). These cell structure surfaces are essential in different stages of biofilm formation. Besides these, secretion systems,

especially type III secretion system (T3SSs), are the multi-protein organelles that span the bacterial cell envelope. Though they are evolutionarily-related to flagella, and are essential to deliver effector proteins into the host cells (Buttner, 2012), which modulates host cell properties to enable bacterial survival in the host cell and thus plays a role in pathogenesis.

Overall, the bacterial cell envelope is very critical as it performs various essential roles including bacterial protection from environmental assaults, serves as an organelle for energy production, energy transport and ATP synthesis in association with bacterial membrane. Two of the envelope stress response systems that are activated in response to the stress-mediated envelope damage are RpoE and Cpx systems.

2.2. The Extracytoplasmic Stress Response (ESR) System

The Extracytoplasmic Stress Response (ESR) System monitors defects or damages to the cell envelope due to environmental perturbations and restores homeostasis by altering the transcriptome in a manner that favors bacterial survival in harsh environment. By the complexity of the bacterial envelope, there are several ESRs that function as an interconnected safety net to repair or modify damage to the cell envelope; two of these ESRs i.e., the RpoE and Cpx systems are described below in more detail.

2.2.1. The RpoE Stress Response System

Bacterial sigma factors enable RNA polymerase to bring about the transcription of specific genes. These sigma factors belong to either sigma 70 or sigma 54 families. The sigma 70 family can be broadly divided into four groups based on their gene structure and functions. Group 1 are closely related to sigma 70 of *Escherichia coli*. Group 2 proteins are also closely related to sigma 70, however they are not essential for bacterial cell growth. Group 3 are more distantly related to sigma 70 and are essential in response to specific signals. Lastly, Group 4 consists of highly diverged extra-cytoplasmic function (ECF) sub-family, which sense and respond to signals from the extracytoplasmic environment (Paget & Helmann, 2003). RpoE sigma factor (σ^E) belongs to the extra-cytoplasmic function sub-family which enables RNA polymerase to initiate transcription of genes involved in cell envelope biogenesis. Along with *rpoE*, there are three other downstream genes, *rseA*, *rseB* and *rseC*, in the *rpoE* operon (Tam *et al.*, 2002; Collinet *et al.*, 2000). RpoE activation is mostly associated with misfolding of the outer membrane proteins (Raivio & Silhavy,

1999). Various stress conditions are associated with RpoE activation. One of the conditions shown to stimulate the σ^E response is heat stress, wherein cells are subjected to temperatures above 42°C. This heat shock results in misfolding of proteins which subsequently leads in activation of RpoE (Erickson & Gross, 1989). Other factors known to activate the σ^E response includes the overproduction of outer membrane protein C (OmpC) or other outer membrane proteins (OMPs) (Mecsas *et al.,* 1993). The RpoE response is also important in pilus assembly, for example, σ^E senses and responds to the production of the misfolded P-pilus subunit, PapG, in the absence of the PapD chaperone (Jones *et al.,* 1997). In addition, oxidative stress, cold shock, exposure to various detergents, osmotic stress, starvation, stationary phase, and light can also cause activation of σ^E [review article by Rowley *et al.* (2006)].

2.2.1.1. Regulation of the RpoE System

***Structure of RpoE sigma factor* (σ_E):** The sigma factors consists of four, flexibly-linked structural domains, $\sigma_{1.1}$, σ_2, σ_3, and σ_4 that contain the conserved regions 1.1, 1.2-2.4, 3.0-3.1 and 4.1-4.2, respectively (Campbell *et al.,* 2002; Malhotra *et al.,* 1996;Vassylyev *et al.,* 2002). Many sigma factors also contain a non-conserved region (NCR) that is inserted between regions 1.2 and 2.1. However, ECF sigma factors are evolutionarily-different from σ_{70} proteins. Out of the four conserved regions observed in sigma factors, the N-terminal subdomain $\sigma_{1.1}$ and part of σ_3 are missing in ECF sigma factors, including σ_E. The σ_E structure consists of an N-terminal domain corresponding closely to the structure of the conserved σ_2 domain of the primary sigma factors, followed by a 26-residue flexible linker, and a C-terminal domain which corresponds to the conserved σ_4 domain of the primary sigma factors [review by Brooks & Buchanan (2008)]. These domains of σ_E (i.e., σ_2 and σ_4) are highly conserved. Generally, σ_2 and σ_4 are responsible for recognizing the -10 and -35 regions of the promoter, respectively. Figure 2.2.1 shows the comparison between the domain structure of Group 1 sigma 70 factors and ECF sigma factors.

Anti-sigma factor RseA: RseA is an inner membrane protein with the N-terminal portion in the cytoplasm and the C-terminal portion in the periplasm (Missiakas *et al.,* 1997). In the absence of envelope stress, σ_E is bound to anti-sigma factor RseA, inhibiting the σ_E activity by interfering with the formation of the σ_E-RNA polymerase complex (Campbell *et al.,* 2003). In the presence of stress

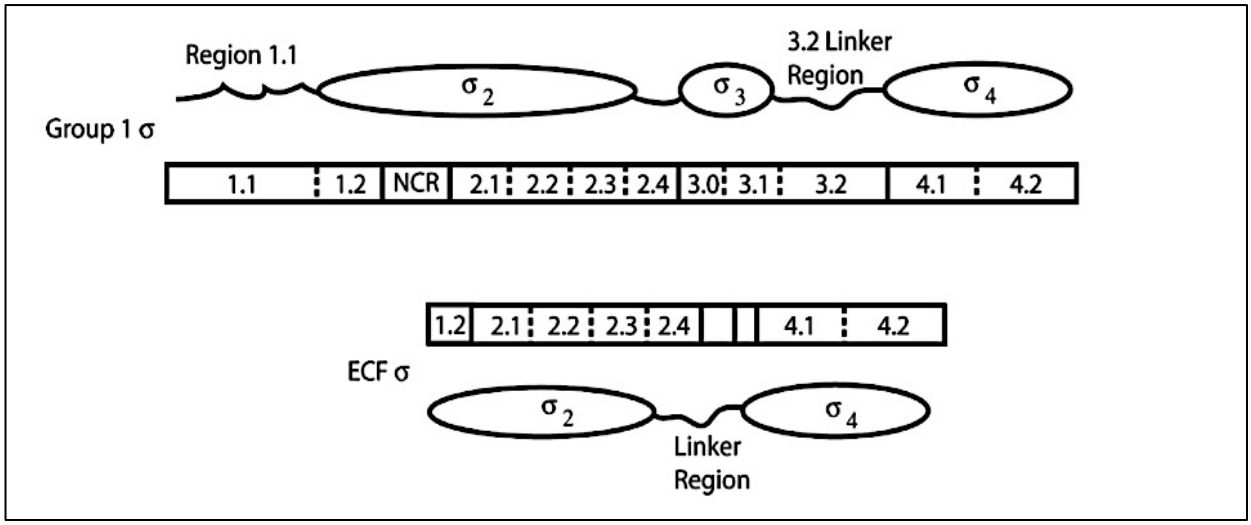

Figure 2. 2. Comparison of the domain structures of ECF sigma factors and Group 1 sigma 70 factors. The 1.1 region, non-conserved region (NCR), and much of σ_3 are missing from ECF sigma factors. The ECF sigma factors only have two globular domains, σ_2 and σ_4, which are responsible for recognizing the -10 and -35 regions of the promoter, respectively. Adapted from Brooks & Buchanan (2008).

that results in misfolding of outer membrane proteins (OMPs), a series of regulated proteolysis steps occurs that release the σ_E from the RseA and promotes transcription. Details of these steps are discussed below.

Regulation of DegS proteolysis of RseA: A cascade of events is initiated that results in the regulated proteolysis of the RseA, thus releasing the σ_E from RseA. This process is well-characterized and it involves two molecular signals: the presence of misfolded outer membrane proteins and LPS biosynthesis intermediates in the periplasm (Lima *et al.,* 2013). In the presence of misfolded OMPs in the periplasm, DegS, an oligomeric serine protease part to Htr family, cleaves the periplasmic domain of RseA (Kim & Kim, 2005; Clausen *et al.,* 2002). One feature of the Htr family protease is the presence of a PDZ domain. PDZ domain (named after three PDZ-containing proteins: the postsynaptic protein PSD-95/SAP90, the *Drosophila* septate junction protein Discs-large, and the tight junction protein ZO-1) are modular protein interaction domains that play an essential role in protein targeting and assembly (Ponting., 1997). The PDZ domain is typically 80-90 amino acids arranged as 6-β strands and 2-α helices (Javier & Rice, 2011). In absence of stress, the YxF peptide motif is buried between two β-strands of the properly-folded OMPs; however, in the presence of stress, the improper folding of OMPs makes the YxF peptide motif accessible which is sensed by PDZ domain activating the protease domain, causing DegS to cleave RseA (Cowan *et al.,* 1995; Walsh *et al.,* 2003). Along with misfolding of OMPs, a second signal (i.e. presence of LPS biosynthesis intermediates) is also required that is sensed by RseB which protects RseA from proteolysis (Lima *et al.,* 2013). The lipid A component of the LPS compounds containing the phosphorylated N-acetylglucosamine disaccharide and two N-linked acyl chains bind to RseB, causing it to dissociate from RseA (Lima *et al.,* 2013).

Regulation of RseP and proteolysis of RseA: Following the cleavage of the periplasmic domain of RseA via DegS, RseP, a zinc metalloprotease belonging to the S2P group of intramembrane proteases, cleaves the cytoplasmic domain of RseA thus releasing the σ_E-RseA inhibitory complex into the cytoplasm (Akiyama *et al.,* 2004; Kanehara *et al.,* 2002; Alba *et al.,* 2002). The σ_E-RseA inhibitory complex binds to SspB (stringent starvation protein B) which activates ATPase activity of ClpX and directs the complex to ClpXP, a protein-degrading machinery complex (Flynn *et al.,* 2004). ClpXP degrades the RseA portion, releasing the σ_E which then binds to the core RNA

polymerase resulting in the transcription of σ_E dependent genes. Figure 2.2.2 provides a schematic model for the σ_E signal transduction pathway in *E. coli.*

2.2.1.1. RpoE Regulon

Studies carried out by Rhodius and his team to develop and validate an accurate promoter prediction model for *E. coli* K-12 and eight other genomes led to the prediction of a total of 89 unique RpoE-controlled transcription units (TU) (Rhodius *et al.,* 2006). The other eight genomes included *E. coli* CFT073, *E. coli* O157, *Salmonella* Typhi, *Salmonella* Typhimurium, *Shigella flexneri*, *Yersinia pestis*, *Photorhabdus luminescens* and *Erwinia carotovora*. The portion of the RpoE regulon that was conserved across genomes was classified as the core RpoE regulon; whereas, the larger variable portion was classified as the extended RpoE regulon. The regulon was predicted to perform pathogenesis-associated functions, suggesting that RpoE performs organism-specific functions necessary for organism-host interactions.

The core RpoE regulon

The core RpoE regulon consists of 19 transcriptional units (TU) and 23 proteins, of which 20 proteins have known functions. Interestingly, the genes identified in the core RpoE regulon are involved in ensuring synthesis, assembly, and homeostasis of LPS and outer membrane porins. Five of the members identified as a part of the RpoE core regulon are involved in synthesis and assembly of lipopolysaccharides (LPS). LpxA, LpxB, LpxD and PlsB promote the synthesis of Lipid A (Raetz & Whitfield, 2002). Lipid A is an essential component of the Gram negative bacterial OM and serves as an anchor for lipopolysaccharides (Fahy *et al.,* 2005). The fifth member in the regulon (BacA) contributes to the LPS assembly (El Ghachi *et al.,* 2004). The other six members of the RpoE regulon promote OMP assembly. Skp, FkpA, and DegS exhibit chaperone activity, YfiO is essential for cellular activity, YraP promotes the assembly of porins and other outer membrane proteins and YaeT function in complexation with three lipoproteins (YfiO, YfgL and NipB) to form a part of a complex that inserts OMPs into the OM (Onufryk *et al.,* 2005; Wu *et al.,* 2005; Rizzitello *et al.,* 2001). Furthermore, the core RpoE regulon also includes FtsZ which is involved in initiating cell division (Erickson *et al.,* 2010).

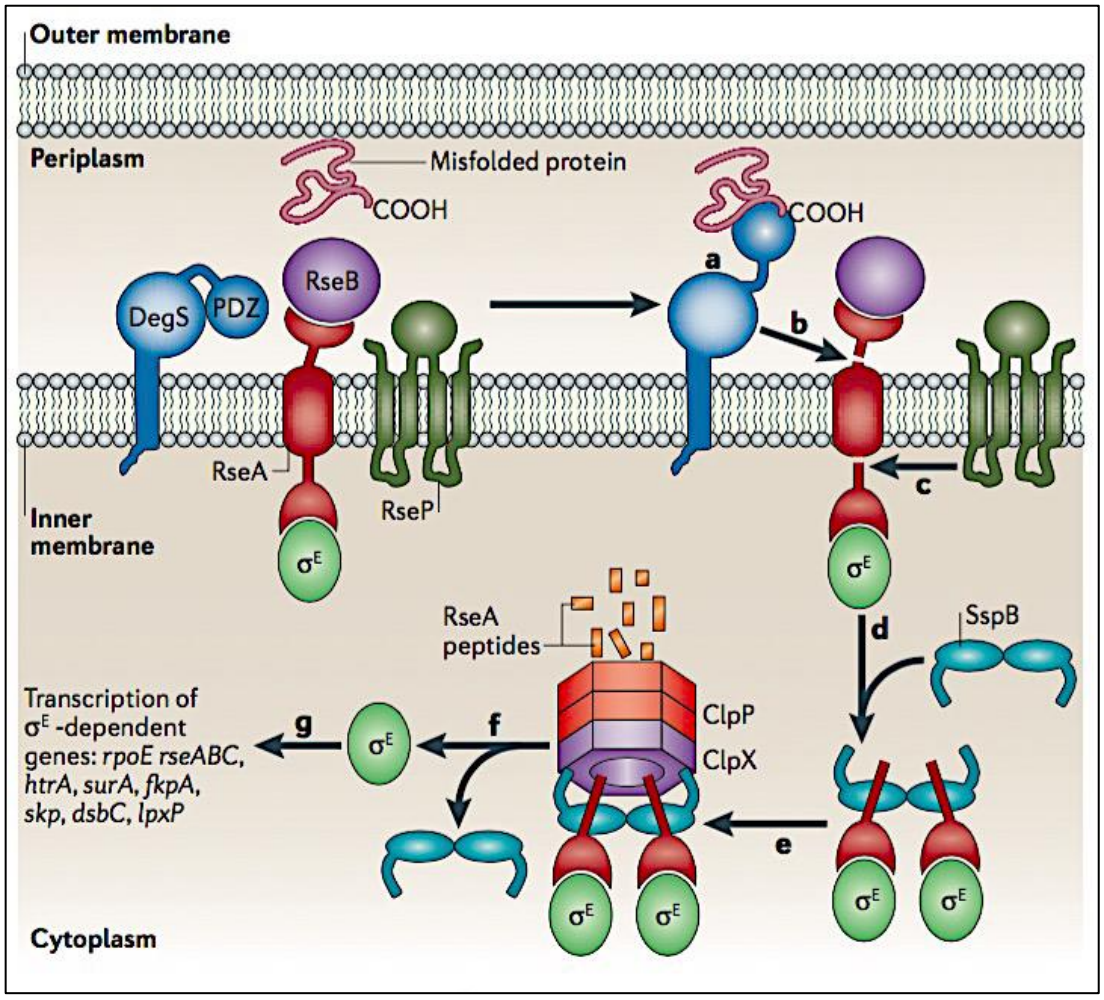

Figure 2. 3. Signal transduction pathway for σ_E activation in response to extracytoplasmic stress. In the absence of misfolded proteins, the PDZ domain of DegS prevents degradation of RseA at periplasmic cleavage sites. However, in the presence of misfolded outer-membrane proteins (OMPs), the PDZ domain of DegS interacts with the carboxyl end of the OMPs (a). This activates DegS protease activity, which results in the cleavage of at its periplasmic site (b). RseA is cleaved at cytoplasmic end by RseP protease following the cleavage by DegS (c), σ_E-RseA inhibitory complex is released into the cytoplasm, following which it binds to SspB (d), SspB directs this complex to ClpXP (e). ClpXP specifically degrades the RseA portion, leading to release of σ_E (f). This allows σ_E to bind to core RNA polymerase with helps in the transcription of σ_E -regulated genes (g). Adapted from Rowley *et al.* (2006).

The extended RpoE regulon

The extended RpoE regulon consists of more than 60 unique RpoE-controlled TU present in fewer than six of the nine genomes (Rhodius *et al.,* 2006); most of the predicted RpoE regulon members are predicted to be involved in pathogenesis. However, these members were not conserved in all the 9 genomes scanned. This could possibly be because the response of RpoE may be organism-specific which might facilitate infection/invasion for a particular niche of the bacterium. Thus, the extended RpoE regulon is not very well-defined and reflects the lifestyle of different bacterial species. Hence, it would be interesting to elucidate the wide range of genes regulated by RpoE and its role in the assembly of OMPs and pathogenesis in different organisms.

2.2.1.2. RpoE Role in Stress Response

I. Role of RpoE in Antimicrobial resistance (AMR)

Studies have linked the role of RpoE in antimicrobial resistance (AMR). An increase in the expression of RpoE was observed when exposed to antimicrobial peptides like BPI-derived peptide P2 and α-defensing cryptdin-4 (Crp4) that target the cell envelope in *S.* Typhimurium indicating the role of RpoE in protection against antimicrobial peptides (Crouch *et al.,* 2005). Also, the *rpoE* null mutants of *S.* Typhimurium were sensitive to cationic antimicrobial peptides (CAPs) like polymyxin B (Humphreys *et al.,* 1999). A growth phase-dependent change in *rpoE* deleted strain of *E. coli* O157 in tolerance to chlorhexidine, a polycationic antimicrobial agent, was recently reported (Vidovic *et al.,* 2018). Dependence on the functionality of the RpoE was seen upon entry into the stationary phase where both the WT and the *rpoE* mutant strain underwent growth arrest with the *rpoE* mutant strain was more sensitive compared to WT strain of *Escherichia coli* (Vidovic *et al.,* 2018). In addition, *rpoE* mutation in *S.* Typhi conferred reduced resistance to wide range of antibiotics, including β-lactams, quinolones and aminoglycosides (Xie *et al.,* 2016). Thus, a clear link is evident between RpoE sigma factor, envelope stress and antimicrobial resistance.

II. Role of RpoE in pathogenesis

RpoE sigma factor plays a central role in pathogenesis by protecting bacteria from a range of envelope stresses. The role of RpoE in bacterial pathogenesis is complex and varies from one pathogen to another. It might play a vital role in one organism but may not have a significant role in pathogenesis in another. Also, the response of RpoE may vary depending on host-pathogen interactions. Some species-dependent features in RpoE's role in pathogenesis are provided below.

Salmonella, an invasive pathogen, requires RpoE for its survival in macrophages *in vitro* and also in host tissue *in vivo* (Humphreys *et al.,* 1999; Eriksson *et al.,* 2003; Crouch *et al.,* 2005). RpoE positively-regulates the expression of *htrA*, which has been found to be important gene for virulence in a wide range of Gram negative and Gram positive bacterial pathogens (Humphreys *et al.,* 1999). Studies on *Haemophilus influenza* and *Mycobacterium tuberculosis* have shown that RpoE is highly-expressed in macrophages and is essential for bacterial survival in those phagocytic cells (Craig *et al.,* 2002; Manganelli *et al.,* 2001). However, it was also observed that *rpoE* mutants were able to survive in macrophages but mutants exhibited decreased survival in the environment in the case of both *H. influenza* and *M. tuberculosis* (Craig *et al.,* 2002; Manganelli *et al.,* 2001). RpoE sigma factor is essential in *Vibrio cholera* to colonize the intestinal tract of infant mice. The role of RpoE is more prominent in survival of the pathogen within host at post-invasion stages of diseases. Thus, in case of *V. cholera*, the *rpoE* mutant does not affect the expression of the toxin co-regulated pilus (TCP) that is required during initial stages of infection (Kovacikova & Skorupski, 2002); whereas, the Cpx system (described in detail below) is more involved during the initial stages of infection.

III. Role of RpoE in biofilm formation

The role of RpoE sigma factor in biofilm formation is highlighted in the study on Crohn's Disease Associated Adherent-Invasive *E. coli*. It was observed that inhibition in RpoE sigma factor pathway resulted in a decreased adherence and biofilm formation (Chassaing & Darfeuille-Michaud, 2013). In *Streptococcus pneumoniae* it was seen that mutation in *rpoE* gene enhanced biofilm development (Churton *et al.,* 2016). Thus, there exists some evidence to link the RpoE sigma factor with biofilm formation; however, the precise role of RpoE sigma factor remains unclear. Accordingly, it would be interesting to identify the positive or negative role of RpoE sigma factor in biofilm formation in various bacteria, and in particular, within NTS.

2.2.2. The Cpx Stress Response System

A second commonly-used bacterial system to sense and respond to envelope perturbations is the Cpx (Conjugative Plasmid Expression) system. The Cpx system is a two-component signal transduction system (TCST) composed of a transmembrane sensor histidine kinase and a cytoplasmic response regulator. The *cpx* locus contains two genes, *cpxA* and *cpxR*, that encode for histidine kinase (HK) and response regulator (RR) of the TCST, respectively (Danese *et al.,* 1995;

Raivio & Silhavy, 1997). The *cpx* locus is found in several gamma-proteobacteria. Conditions that are required for the activation of the Cpx pathway are diverse. Some of the conditions known to activate the Cpx pathway include alkaline pH, EDTA, growth, chloride ions, indole, copper, and ethanol. Other factors include the over-expression of misfolded pilus proteins, high osmolarity, over-expression of misfolded periplasmic proteins that aggregate in the inner membrane, adherence to abiotic surfaces, presence of aminoglycoside antibiotics and alterations in phospholipid ratios. Deletion of efflux pump components, mammalian peptidoglycan recognition proteins, spheroplasting, mutation in the gene encoding for the inner membrane protein localization, assembly of type IV secretion system, disulphide bond disruption in the periplasm, accumulation of enterobacterial common antigen assembly intermediates are also known to activate the Cpx pathway [from review by Raivio (2014)].

***Over-expression of outer membrane lipoproteins (e.g. NlpE)*:** One of the major inducing factors for Cpx activation is the over-expression of NlpE lipoprotein. An NlpE-dependent activation of the Cpx system was reported in *E. coli* required for adhesion to abiotic surfaces (Snyder *et al.,* 1995). The role of NlpE has been linked to biofilm formation in *Acinetobacter baumannii* (Siroy *et al.,* 2006). Also, NlpE has also been characterized as a copper homeostasis protein (Gupta *et al.,* 1995). However, the exact mechanism for the activation of Cpx pathway by NlpE over-expression is not known; but, NlpE over-expression results in protein mislocalization and misfolding which might activate the Cpx system.

***Over-expression of misfolded pilus proteins*:** In uropathogenic *E. coli*, and in enteropathogenic *E. coli*, Pap pilus (bundle forming pili) and BfpA (a major subunit of the type IV bundle forming pilus) are shown to activate the Cpx system, respectively. It is assumed that misfolding of Pap and BfpA subunits activates the Cpx system. Cpx responds by reducing the stress associated with pilus over-expression and also might facilitate efficient pilus assembly by assisting protein folding and degrading mislocalized intermediates (Nevesinjac & Raivio, 2005; Vogt *et al.,* 2010). It is interesting since both Pap and NlpE play an essential role in adherence to host cells, and all are linked to the activation of the Cpx system. This suggests that Cpx might have role in biofilm formation and pathogenesis. It is further interesting to note that in the absence of chaperones, pili are not made and misfolded subunits aggregate in the periplasmic space along the IM. Also, the over-expressed NlpE similarly accumulates at the IM (Raivio *et al.,* 2013). Recent Cpx regulon analyses demonstrated a close association between Cpx response and the IM (Raivio, 2014).

Comparative transcriptomic study in *E. coli* showed a closer relation of Cpx system to the Psp (phage shock protein) and Bae (bacterial adaptive response) stress response systems (Bury-Moné *et al.*, 2009). Evidence relating the Psp response to insults occurring at the IM suggest that proton motive force alterations may be involved (Model & Jovanovic, 1997); whereas, Bae senses toxic compounds and metals via the predominant up-regulation of efflux pumps (Rowley *et al.*, 2006). The above observations suggest that Cpx signaling may respond to toxic compounds and metals in the inner membrane generated due to misfolding of proteins.

***Alkaline pH*:** Elevated pH is another inducing factor for the activation of Cpx response, as demonstrated in *E. coli* and *Shigella* species (Danese & Silhavy, 1998; Nakayama & Watanabe, 1995). Strains with mutations in the *cpx* locus have been shown to be hypersensitive to alkaline pH. It was also seen that in *Shigella*, *cpxA* mutation altered gene expression of the virulence regulator, *virF*, in a pH-dependent manner. Again, the exact mechanism for the pH-induced Cpx response is unknown; however, it could be possible that structural damage to the envelope occurs as a result of protein denaturation (Nakayama & Watanabe, 1995).

***Growth*:** Growth is one of the known inducing cues for the Cpx pathway. A study on *Yersinia enterocolitica* showed the importance of controlled activation of Cpx system was essential for growth (Ronnebaumer *et al.*, 2009). Studies have also revealed the Cpx pathway is more active in the stationary phase. It was seen that in some strains of *E. coli* K12 that auto-activation of the Cpx operon takes place in the stationary phase in combination with the stationary phase sigma factor, RpoS (Wulf *et al.*, 1999). Another study showed that the Cpx response up-regulated the expression of RprA sRNA, which was linked to growth phase as a regulator for RpoS, and over-expression of RprA thereafter inhibited the Cpx response (Majdalani *et al.*, 2001). Thus, while the varied functions of Cpx remains unclear, it would be interesting to understand its role in the different stages of bacterial growth.

***Alteration in membrane structure*:** Studies have shown that mutants lacking phosphatidylethanolamine (PE), a chaperone for some envelope proteins, strongly activate the Cpx response (Mileykovskaya & Dowhan, 1997), probably due to misfolded proteins in PE mutants. In another study, accumulation of enterobacterial common antigen intermediate lipid II in the inner membrane also activated the Cpx system (Danese *et al.*, 1998).

2.2.2.1. Regulation of the Cpx System

Cpx response is mediated by TCST mechanisms where CpxA is known to be a membrane sensor that can sense the stress and CpxR is a response regulator (Raivio & Silhavy, 1997; Fleischer *et al.*, 2007). Classically, envelope stress signals are transmitted through CpxA to CpxR via phosphotransfer reactions. The existing literature shows that CpxA consists of transmembrane domains with a periplasmic part that acts as a sensory domain which might directly senses stress on the envelope. In the absence of inducing cues (some of which are mentioned above), CpxA functions as a phosphatase, maintaining CpxR in an unphosphorylated state. However, in presence of inducing cues, the auto-phosphorylation of CpxA takes place at the histidine residue. Once phosphorylated, CpxA transfers the phosphate ion to the conserved aspartate residue on the cognate response regulator, CpxR, which then binds to the promoter of the genes under Cpx regulon. A recent study on the structure of CpxA sensory domain in *Vibrio parahaemolyticus* showed that CpxA uses a Per-Arndt-Sim (PAS) domain to sense the signals (Kwon *et al.*, 2012). The mutations in the sensory domain activates CpxA indicating the disruption in the proper folding of the sensory domain directly activates the kinase activity of CpxA (DiGiuseppe & Silhavy, 2003; Raivio & Silhavy, 1997). Besides, two of the signalling proteins that are shown to control the Cpx system include the positive regulator NlpE (described in section 2.2.2) and the negative regulator CpxP (Snyder *et al.*, 1995; Danese & Silhavy, 1998). CpxP encodes a small periplasmic protein, which when over-expressed leads to inhibition of Cpx-regulated gene expression (Raivio *et al.*, 1999). In addition to its inhibitory role on CpxA, CpxP is thought to be a proteolytic adapter protein that binds misfolded proteins and targets them toward DegP, a periplasmic protease (Zhou *et al.*, 2011; Hung *et al.*, 2001). Since CpxP binds to the misfolded protein and the periplasmic sensory domain of CpxA, the Cpx system is in its "on" state. The genes regulated by Cpx system might help to clear misfolded proteins and once the process is completed, the excess CpxP will inhibit the system. However, the exact signal transduction of the Cpx system is not fully understood and there could be various other genes which might also play a role in the Cpx signal transduction pathway. Figure 2.2.3 provides a schematic overview of the Cpx signal transduction pathway.

2.2.2.2. Cpx Regulon

Detailed analysis of the Cpx regulon may provide insights on Cpx-regulated genes and could also help delineate the role of the Cpx response in detail. However, there are only 5 published

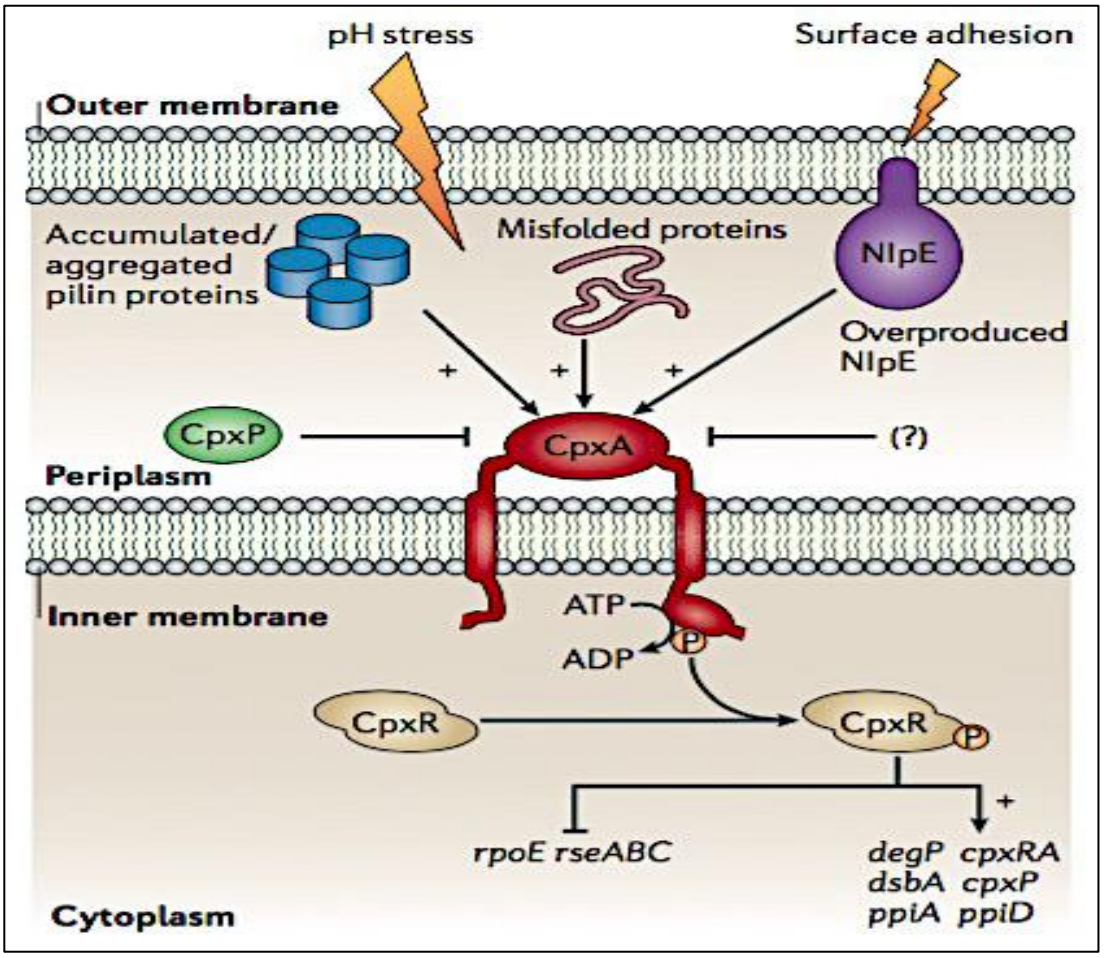

Figure 2. 4. Signal transduction pathway for Cpx activation in response to extracytoplasmic stress. In presence of inducing cues, they are sensed and transduced, likely through the periplasmic domain of CpxA. This activates CpxA kinase activity, which triggers the auto-phosphorylation of a conserved histidine residue in the cytoplasmic domain. Phosphorylated CpxA then transfers the phosphate group to an aspartate residue in the N-terminal domain of the CpxR, a response regulator. CpxR-P then binds to specific sites on DNA. CpxP-R binding results in up-regulation or down-regulation of specific genes in the Cpx regulon. One of the gene up-regulated by CpxR-P is *cpxP*, which encodes a small periplasmic protein, CpxP, that binds to CpxA, inhibiting its kinase activity and thus inhibiting the Cpx stress signaling system. However, in presence of the envelope stressors CpxP dissociate from CpxA thus activating the Cpx system. Adapted from Rowley *et al.* (2006).

studies that examined the Cpx regulon in detail. Transcriptomic analysis of the Cpx regulon was carried out in *E. coli, Haemophilus ducreyi* and recently *in V. cholera* (Labandeira-Rey *et al.,* 2010; Gangaiah *et al.,* 2013; Bury-Moné *et al.,* 2009; Raivio *et al.,* 2013; Acosta *et al.,* 2015). Initial studies of the Cpx regulon identified three genes regulated by CpxR; *dsbA* (encoding major periplasmic disulfide oxidoreductase), *ppiA* (encoding peptidyl propyl isomerase) and *degP* (encoding periplasmic chaperone) (Danese & Silhavy, 1997; Danese *et al.,* 1995; Pogliano *et al.,* 1997). These three genes function in the proper folding of cell envelope. Further analysis of the upstream region of these genes helped in identifying a consensus binding site for CpxR. The binding site for CpxR was identified as 5' GTAA-NNNNNNN-GTAA-3', where N is any nucleotide. This provided additional evidence used to identify Cpx-regulated genes (De Wulf *et al.,* 2002). Further, *psd* and *secA* (genes encoding proteins affecting biogenesis of phospholipids and proteins involved in bacterial envelope, respectively) are also proposed to be regulated by the Cpx system. The *spy* gene (with putative function in outer membrane biogenesis) was shown to be up-regulated by conditions activating the Cpx response (Raivio *et al.,* 2013). Besides the genes associated with envelope protein folding that provided strong evidence of Cpx regulation, genetic microarray analysis revealed that the Cpx regulon contains several hundred genes that were up- and down-regulated, indicating the diverse role of Cpx in various cellular processes. A bioinformatics study was carried out to identify the CpxR DNA binding site in *E. coli* K-12 strain MG1655 (Liu & De Wulf, 2004). The CpxR binding site was identified at a location upstream of various genes with roles in protein folding, stress adaptation, motility, biofilm formation and pathogenesis. Recent analysis of the Cpx regulon in *E. coli* has shown that more than 100 proteins respond to certain envelope stressors in a Cpx-dependent manner (De Wulf *et al.,* 2002).

Another group of genes proposed to be regulated by the Cpx system are outer membrane proteins like OmpC, OmpF and NanC which are involved in transport (Batchelor *et al.,* 2005). Also, genes encoding efflux pumps, like *acrD* and *mdtA,* are regulated in part by the Cpx system (Hirakawa *et al.,* 2005). It has been shown that mutation in the *cpx* locus leads to an increase in *ompC* expression with a concurrent decrease in *ompF* expression. Furthermore, *cpxR* mutations decrease the activity of the *nanC* gene. However, the aforementioned genes are also strongly-regulated by other stress response pathways. For example, *ompC* and *ompF* have been shown to be regulated by EnvZ- OmpR signal transduction pathway, and the *acrD* and *mdtA* are regulated by the Bae system. However, the CpxR binding site has been shown to overlap with upstream regions

of both OmpR and BaeR (Price & Raivio, 2009). Recently, sRNA components of the Cpx response was recognized and it was shown that CpxR increases the level of sRNAs OmrA, OmrB and MicF which is known to be regulated by EnvZ- OmpR signal transduction pathway (Raivio *et al.,* 2013; Guillier & Gottesman, 2006; Vogt *et al.,* 2014). Cpx induces these sRNAs by producing an IM protein MzrA, which stimulates the EnvZ histidine kinase and links Cpx response to EnvZ-OmpR (Gerken *et al.,* 2009; Gerken & Misra, 2010). Also, an another sRNA RprA which is highly-induced component of Rcs response, plays an important role in biofilm formation (Majdalani & Gottesman, 2005). The CpxR was shown to bind to the promoter of *rprA* and increase its production thus highlighting the interconnection of these pathways with each other (Vogt *et al.,* 2014). Overall, the evidence available to date suggest that these pathways are somehow linked; clearly, it would be interesting to elucidate the relationship between these pathways. The Cpx response is said to negatively-regulate the RpoE response. Studies have shown that CpxR-P binds upstream of *rpoE* gene which encodes RpoE and results in a reduction in the RpoE response (Miticka *et al.,* 2003). Genes involved in the production or function of extracellular bacterial structures such as flagella, and/or pili are also believed to be regulated by the Cpx system. Thus, the Cpx regulation of genes with diverse functions suggests that Cpx response might affect a variety of complex bacterial behaviors.

2.2.2.3.Role of Cpx in Stress Response

I. Role of Cpx system in resistance to antimicrobial agents

The role of the Cpx response in regulating IM-associated processes have been studied and the link between the Cpx response to the IM condition is highlighted in the above text. Recent insights into Cpx regulation of the IM indicates its plausible role in antibiotic resistance and efflux, based on the observation made on the function of the *cpx* locus. The Cpx response has been prominently-linked to resistance to broad range of antibiotics in a diverse group of bacteria. Recent studies on the role of Cpx-regulated genes involved in conferring resistance to bacteria, are discussed below:

In *E. coli*, Cpx response activation has been linked to resistance to β-lactam, fluoroquinolone, and aminoglycoside antibiotics as well as to deoxycholate, copper, indole, and the cationic antimicrobial peptide (CAMP), protamine, as a result of increased expression of *tolC* and the MDR efflux pumps, *mdtABC* and *acrD* (Weatherspoon-Griffin *et al.,* 2014; Nishino *et al.,*

2010).The role of the Cpx response in antibiotic resistance is not yet fully-elucidated, but it is proposed that Cpx might have role in antibiotic resistance under certain conditions.

In pathogenic organisms like *V. cholera* and *Klebsiella pneumoniae*, MDR efflux pumps (transport systems for exporting a wide range of substances, like antibiotics, dyes, detergents and metabolites) is Cpx system-regulated and has been linked to antibiotic resistance. In *V. cholera*, it has been observed that activation of the Cpx system enhances resistance to ampicillin, bile salts, and other detergent-like compounds (Slamti & Waldor, 2009). In *K. pneumoniae*, a reduction in expression of MDR efflux pumps was seen in *cpxR* and *cpxA* mutants. Also, the mutant was more sensitive to bile, the disinfectant chlorhexidine, as well as various other antibiotics, as compared to WT (Srinivasan *et al.*, 2012). These observations show linkages between the Cpx response and AMR in a diverse group of organisms.

Studies on *Salmonella* and *Pseudomonas spp*. have shown the sensitivity of amidase (an enzyme required during cell division) mutants to vancomycin, detergents, protamine, and multiple antibiotics due to defects in the permeability of the outer membrane (Yakhnina *et al.*, 2015). However, activation of the Cpx response decreases the sensitivity of amidase mutants to protamine, gentamycin, and vancomycin, and may possibly be involved in restoring outer membrane integrity; but, the exact mechanism of action is not known (Weatherspoon-Griffin *et al.*, 2011; Yakhnina *et al.*, 2015). Furthermore, a study carried out by Silhavy and coworkers (Mahoney & Silhavy, 2013) showed that activation of the Cpx system conferred resistance in *E. coli* to hydroxyurea and aminoglycoside antibiotics. In the work involving *P. aeruginosa*, a double *yccA* and *htpX* mutant along with an additional gene of unknown function, conferred greater sensitivity to the aminoglycoside (Hinz *et al.*, 2011). HtpX is a membrane-bound protease and YccA is an inhibitor of the membrane-bound protease, FtsH. In *E. coli*, *htpX* and *yccA* are both Cpx-regulated. Taken together, some of these studies suggest that the Cpx response both limits the permeability of the outer membrane and upregulates the expression of inner efflux pumps important for flushing the cells of antibiotics and other toxic metabolites.

II. Role of Cpx response in pathogenesis

The exact role of the Cpx response has not been investigated during infection events, but the Cpx systems role has been implicated in expression of virulence genes, including pili/fimbriae and the type III secretion system. The following sections provide a description of some of the information that is available with regard to the Cpx response in select bacteria.

Escherichia coli: The role of Cpx response in *E. coli* for the production of pili and fimbriae expression has been investigated and it was seen that inactivation of the Cpx response adversely affects the assembly of some pili. For example, mutation in the *cpxR* gene in *E. coli* K-12 results in production of shorter pili and a higher proportion of cells that do not express any pili (Hung *et al.*, 2001). Similarly, in enteropathogenic *E. coli*, expression of type IV bundle-forming pili (BFP) and adherence to human cells, was affected by mutation in the *cpxR* gene (Nevesinjac & Raivio, 2005). Also, the Cpx response might enhance virulence by increasing the expression of DsbA (periplasmic protein folding factor) and PpiA (peptidylprolyl isomerase A) required for assembly of cell surface structures like pili. DsbA plays a key role in pathogenesis, especially in *E. coli*, and its inactivation affects virulence in several organisms. In *E. coli*, DsbA has been identified as a key virulence factor (Nevesinjac & Raivio, 2005). In *P. aeruginosa*, *dsbA* expression was also shown to become up-regulated upon conversion from the non-mucoid to mucoid condition. DsbA is also essential for proper assembly of pili in *E. coli.* Thus, in *E. coli*, the Cpx response has both positive and negative effects on virulence.

Salmonella serovars: In *S.* Typhimurium, the Cpx system affects invasiveness in a pH-dependent fashion. HilA, an important regulator of the *Salmonella* pathogenicity island 1 (SPI-1) gene expression, is known to be affected by the Cpx response. A study examining a *cpxA* mutant of *S.* Typhimurium showed that *hilA* expression was reduced by 10% at pH 6.0 (as compared to WT strain), but no difference was seen at pH 8.0. It was also seen that at pH 6.0, the invasiveness of *S.* Typhimurium became reduced (Nakayama *et al.*, 2003). However, at alkaline pH of 8.0, *cpxA* and *cpxR* mutants were slightly more invasive that was the wild type. In addition, the *cpxA* mutation affected adherence of *S.* Typhimurium to the host cells (Humphreys *et al.*, 2004). It was also seen that the survival and replication of *S.* Typhimurium within macrophages, *in vitro*, did not require a functional Cpx system.

Shigella spp: The Cpx response controls the expression of two important virulence regulators, VirF and InvE in *Shigella* spp. VirF activates the transcription of *invE*, which, in turn, activates the transcription of TTSS genes required for invasion into eukaryotic cells (Tobe *et al.*, 1993). The post- transcriptional production of InvE is also increased by Cpx response.

Legionella pneumophila: In *L. pneumophina*, *icm-dot* genes are important for infection in amoebae and macrophages. They are essential components of the type IV secretion system (TFTS) that translocate effector proteins into host cells to facilitate intracellular survival. CpxR has been shown

to positively-regulate the transcription of *icm-dot* genes (Gal-mor & Segal, 2003). However, for survival, growth or infection of *L. pneumophila* within macrophages or amoebae, *cpxR* or *cpxA* mutations have no effect.

Xenorhabdus nematophila: A Gram negative bacterium, shares a synergistic association with the entomopathogenic nematode, *Steinernema carpocapsae*. This mutualistic cooperation between bacteria and nematode results in the killing of a variety of insect hosts. However, inactivation of the Cpx response reduces the ability of *X. nematophila* to both colonize its nematode host and successfully infect an insect host. The genes required for the envelope-localized colonization factors, NilA, NilB, and NilC, might have reduced expression in *cpxR* and *cpxA* mutant strains, thus affecting the colonization of the bacterium (Herbert *et al.*, 2007). In *H. ducreyi*, the role of Cpx in inhibition of expression of various virulence determinants has also been shown (Labandeira-rey *et al.*, 2011). It is known that mutation in *cpx* locus reduces the virulence ability of *H. ducreyi* in human volunteers. In summary, Cpx is involved in regulating the expression of various surface virulence factors such as pili/fimbrae, TTSS and the TFSS. However, to date, the direct requirement of the Cpx response for expression of virulence factor has not been studied. An *in vivo* role for the Cpx system has been shown in only in one organism, *S.* Typhimurium. However, the exact relation of the Cpx system and pathogenesis needs to be explored in more detail to clarify its role in host-pathogen interactions. From the above details provided, RpoE sigma factor and Cpx responses appear to play distinct roles in the infection processes of pathogen bacteria. While the Cpx response has been linked to early events in infection such as adhesion, the RpoE sigma factor response appears to be mainly involved in survival within the host at post-invasion steps of disease.

III. Role of Cpx response in biofilm formation

There is evidence highlighting the role of Cpx system in biofilm formation. Cpx is predicted to play an important role in regulating the expression of several genes involved in biofilm formation. Some of the proteins having a role in biofilm formation are transcriptionally-regulated by the Cpx response: i) *flhDC* operon involved in motility encoding the flagellar regulatory complex, FlhDC, is negatively-regulated by the Cpx response. A diminished expression of FlhDC is seen upon activation of the Cpx system (Raivio *et al.*, 2013). Thus, the Cpx system probably contributes to the inhibition of motility which is important in biofilm formation, and ii) Another protein up-regulated by Cpx system is DgcZ, required for controlling the biosynthesis of the

exopolysaccharides essential for surface attachment. Transcription of *dgcZ* is regulated by Cpx system (Lacanna *et al.*, 2016). From the above examples, it seems that the Cpx response in motility inhibition and polysaccharide production could be important for biofilm formation. However, the Cpx response negatively-regulates CsgD, a positive regulator in biofilm formation (Jubelin *et al.*, 2005). CsgD is required for the production of cellulose which in involved in biofilm formation in some bacteria. Also, it was seen that activation of the Cpx response in *E. coli* showed poor biofilm formation (Otto & Silhavy, 2002). Thus, this contrary behavior of the Cpx response in biofilm formation suggests additional work remains to fully-elucidate its role.

2.3. Biofilms

The available literature on envelope stress response systems, especially the RpoE sigma factor and Cpx two-component system, leads to a connecting bridge between biofilm formation and the ESRs. In the below text, I have provided a brief introduction on biofilms and its formation.

2.3.1. Introduction to Microbial Biofilms

Biofilms are one of the most widely distributed and successful modes of life on Earth (Stoodley *et al.*, 2002). They are defined as the aggregates of micro-organisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that adhere to each other and/or surface (Vert *et al.*, 2012). Bacteria in biofilms exhibit a set of "emergent properties" that differ significantly from free-living bacterial cells. These emergent properties of the biofilm communities comprise 'novel structures, activities, patterns, and properties that arise during the process, and as a consequence of self-organization in the complex systems' (Flemming *et al.*, 2016). Bacterial biofilms are widely distributed and play an important role in many industrial and medicinal activities. Biofilms can form on different medical implants such as catheters, artificial hips and contact lens. It has been estimated that biofilms are associated with 65% of the nosocomial infections and the treatment of these biofilm-based infections costs > $1 billion annually (Potera, 1999). Biofilms have a huge impact on food industries affecting the food quality, quantity and the safety of the food products. Besides, biofilms can also cause corrosion of the pipelines and tanks. They also exhibit increased AMR and also enable gene transfer among bacteria which can lead to increase in number of virulent strains (Satpathy *et al.*, 2016).

2.3.2. Biofilm Development

Biofilm formation has been extensively studied for a very long period and in most cases the organisms come together to form a community which gets attached to the surface and is covered by an exopolysaccharide matrix. In biofilm, the microorganisms account for less than 10% of the dry weight whereas the matrix accounts for over 90% (Satpathy *et al.,* 2016). Biofilm formation can be divided into five stages (Satpathy *et al.,* 2016). Step 1: Attachment of planktonic cell with the substrate by adhesion mechanism. The characteristics of the substratum may have a significant effect on the rate and extent of attachment by microorganisms. Besides the characteristics of the substratum, characteristics of the bacterial cell surface also play a crucial role in attachment. For example, a study showed the importance of flagella and fimbriae in biofilm formation (Korber *et al.,* 1989; Rosenberg *et al.,* 1982). Step 2: Cell adsorption and multiplication: The adsorption is followed by passive transport of bacteria mediated by weak long-range forces of attraction. Covalent and hydrogen bonds create strong, short-range forces which result in irreversible attachment (Chandki *et al.,* 2011). Step 3: Early development of biofilm architecture, production of cell-cell signaling molecules. Step 4: Production of firmly-attached, mature biofilm architecture with EPS which is called the dark matter of biofilms because of the large range of matrix biopolymers and the difficulty is analyzing it, and Step 5: Dispersion of cells and cell aggregates from the biofilm. This is an essential stage in biofilm life cycle that contributes to biological dispersal, bacterial survival and disease transmission. This stage is as complex as other stages in biofilm formation which involves numerous environmental signals, signal transduction pathways, and effectors (Karatan & Watnick, 2009). A schematic representation of biofilm formation is represented in Figure 2.3.1.

2.3.3. Genetic Aspect of Biofilm Formation

Bacteria have evolved various structural components that play a critical role in facilitating the sensing and attachment of the bacteria to the nearby surfaces. Some of these include pili, flagella, fimbriae and various secretion systems that have been found to be essential components in biofilm formation in various pathogens. In *P. aeruginosa*, Type IVa pili are required to generate mature biofilm structure (Heydorn *et al.,* 2002; Toole & Kolter, 1998). Besides, a surface-sensing system, the Wsp regulatory system homologous to CheRB encoding protein required for chemotaxis, triggers biofilm formation in *P. aeruginosa* (Huangyutitham *et al.,* 2013; Connor *et al.,* 2012).

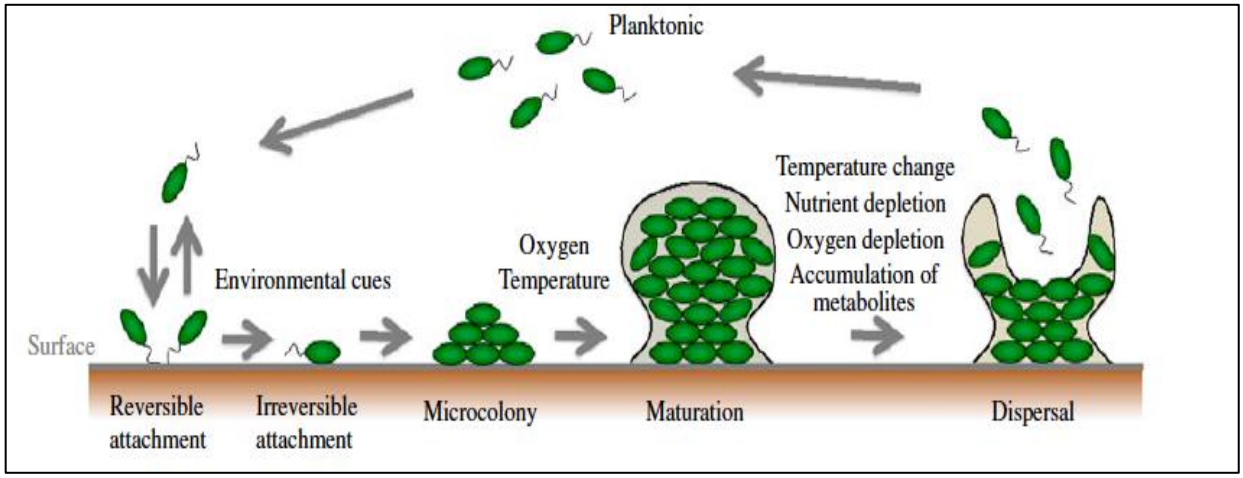

Figure 2. 5. A schematic representation of biofilm formation. Adapted from Toyofuku *et al.* (2016).

Negative regulation of the stationary phase sigma factor, RpoS, has been reported in biofilm formation, where the *rpoS* mutant formed densely-packed biofilm as compared to the wild-type *P. aeruginosa* (Heydorn *et al.*, 2002). Also, reduction in attachment to abiotic and biotic surfaces was observed in an O-polysaccharide-deficient *Pseudomonas* spp. (Dekkers *et al*., 1998; DeFlaun *et al.*, 1999). Inhibition of flagellum rotation increases biofilm formation by upregulating the DegS-DegU two-component system, resulting in expression of BslA required for biofilm formation (Kobayashi & Iwano, 2012). In *V. cholerae*, a condition that leads to inhibition of flagellar motor function enhances biofilm formation and virulence (Gardel & Mekalanos, 1996). Very tight regulation connections between Quorum Sensing, c-di-GMP and sRNA has been proven to be essential in biofilm formation of *V. cholerae* (Srivastava & Waters, 2012). Mutation in *csgA* gene encoding biosynthetic curlin gene and *fimH*, a type I pili biosynthetic gene, results in significant decrease in attachment of *E. coli* (Vidal *et al.*, 1998; Dorel *et al.*, 1999; Pratt & Kolter, 1998). Furthermore, mutation in LPS core biosynthesis genes *rfaG, rfaP* and *galU* also resulted in deficient biofilm formation in *E. coli* (Genevaux *et al.*, 1999; Reniero-Herva *et al.*, 1999). In *Salmonella*, the ability to form biofilms is an important factor for virulence. The biofilm formed by *Salmonella* spp. are often encountered in barns, kitchen, toilets or in gallstones and are consequently becoming a potential threat to our society. The CsgD protein, a master regulator from LuxR family, is essential for biofilm formation in *Salmonella* (Ogasawara *et al.*, 2011). It increases the expression of curli and Bap genes and also indirectly post-transcriptionally activates the cellulose biosynthesis that is required for biofilm formation (Fàbrega & Jordi, 2013). Adhesion-mediated type I fimbriae, Lpf and Pef, are also required for the initial stages of adhesion in *Salmonella* biofilm formation (Ledeboer *et al.*, 2006). The increasing number of *Salmonella* infections as reported by World Health Organization (WHO), and the difficulty in eradicating the biofilms often poses a concern and hence a detailed understanding of complex process of *Salmonella* biofilm formation is crucial.

2.4. Introduction to *Salmonella*

2.4.1. Classification and Nomenclature of *Salmonella* spp.

The *Salmonella* genus belongs to the family of Enterobacteriaceae and is an important pathogen for both humans and animals. The nomenclature for the genus *Salmonella* is complex and has evolved over the past several decades. The current nomenclature system approved and used

by WHO, Centers for Disease Control and Prevention (CDC) and American Society for Microbiology (ASM) includes two species of *Salmonella*, *S. enterica*, the type species, and *S. bongori* (listed in subspecies V) (Su & Chiu, 2007). *Salmonella enterica* can be further divided into six subspecies and these are as follows: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Brenner *et al.*, 2000). *Salmonella enterica* subspecies are differentiated biochemically and by genomic relatedness. The names of serotypes usually refer to the geographical location where the serotype was first isolated. The majority (59%) of the 2,463 *Salmonella* serotypes belong to *Salmonella enterica* subsp. I. Strains in these serogroups cause infection in humans and warm-blooded animals. Serotypes in other subspecies are usually isolated from cold-blooded animals (Farmer *et al.*, 1984). Thus, the complete designation is, for example, *Salmonella enterica* subspecies *enterica* serovar Enteritidis which could be abbreviated to *S.* Enteritidis (as used in this thesis).

Most NTS infections are caused by *S. enterica* subspecies *enterica* serotypes Enteritidis, Typhimurium, Newport, Heidelberg, and Javiana (Brenner *et al.*, 2000). In contrast to typhoid fever, which is common in developing countries, NTS salmonellosis occurs worldwide and is one of the major concerns for developed and developing countries, including BRICS (Brazil, Russia, India, China and South Africa) countries (Gal-mor *et al.*, 2014). NTS is the leading cause of foodborne illness and is responsible for causing an average of 4.07 million Disability Adjusted Life Years (DALYs) between 1990-2012 (Kirk *et al.*, 2015). Estimation of annual costs for salmonellosis has ranged from billions of dollars in the USA to millions to dollars and pounds in Canada and United Kingdom, respectively. Moreover, the increasing antimicrobial resistance found in *Salmonella* has become a global problem that requires urgent attention. Although this organism has been studied intensively, much more work is needed to complete our understanding of this complex group of pathogens.

2.4.2. *Salmonella enterica* serovar Enteritidis

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is one of the most frequently-reported serovar affecting both human and animal health. Interestingly, *Salmonella enterica* serovar Typhimurium was replaced by *S.* Enteritidis by 1990 as the primary cause of salmonellosis in the world (Bäumler *et al.*, 2000; Guard-petter, 2001). Notably, *S.* Enteritidis is the only human pathogen associated with egg contamination, which presents a unique threat to food safety. In the

USA, *S.* Enteritidis has resulted in losses of more than 500 million dollars annually as a consequence of infections in humans and associated medical costs, as well as lost productivity to the egg and meat industries (Frenzen *et al.,* 1999; Fadl *et al.,* 2002). These losses are mainly because of its unique ability to contaminate eggs without causing any visible illness in the infected birds. One of the unique abilities of *S.* Enteritidis is its ability to form biofilm on materials of different nature and under different growth conditions (Korber *et al.,* 1997; Austin *et al.,* 1998; Solano *et al.,* 1998). Biofilm-forming *S.* Enteritidis isolates are considered virulent and their ability thus correlates with enhanced oral invasiveness (Guard-Petter *et al.,* 1996; Solano *et al.,* 2001). The ability of *S.* Enteritidis to withstand these diverse conditions needs in-depth investigation in order to reduce the infection and transmission of this pathogen.

2.4.3. *Salmonella* Pathogenesis

Non-typhoidal salmonellae enters the body usually through ingestion of contaminated food. There are variety of factors that play a crucial role in pathogenesis which includes: 1) the ability to invade cells, 2) a complete lipopolysaccharide coat, 3) the ability to replicate intracellularly, and 4) elaboration of toxin(s). The common virulence trait that contribute to the *Salmonella* pathogenesis include: i) Type III secretion system (TTSS-1) encoded on *Salmonella* pathogenicity island-1 (SPI-1) which mediates invasion, ii) TTSS-2 encoded on SPI-2 which is required for the survival within macrophages, and iii) expression of LPS and flagellin, essential for triggering an Toll-like receptor (TLR)-mediated inflammatory response (Mu *et al.,* 2012). Invasion followed by persistence in the host cell are two of the crucial aspects of pathogenesis in *Salmonella*. During the invasion in non-phagocytic host cells, it induces its own phagocytosis using genes encoding SPI-1 for invasion and penetration into the epithelial cells. The TTSS, a multichannel protein encoded by SPI-1 helps *Salmonella* to inject effectors across the intestinal epithelial cells thus activating the signal transduction pathways. This triggers a reconstruction of actin cytoskeleton of host cell resulting in membrane ruffling from cell surface followed by internalization of the pathogen. Shortly after invasion, *Salmonella* encounters macrophages within gut-associated lymphoid tissue and enter macrophages through macropinocytosis where they can evade the microbicidal functions of the phagocyte and promote survival and replication with the activation of virulence determinants (Ohl & Miller, 2001; Alpuche-aranda *et al.,* 1994). The ability of *Salmonella* to survive within macrophages is essential to establish a systemic infection in the host. After invasion, *Salmonella*

has to survive in the hostile environment with low pH and avoid lysis by bile salts in upper intestine which is considered essential, although not much information is available on its ability to survive in such hostile intracellular environment despite the identification of a variety of virulence genes [review by Eng *et al.* (2015)]. Most of these virulence determinants for invasion, survival and extra intestinal spread are located in SPI [review by Eng *et al.* (2015)]. Initial host responses involve neutrophil infiltration, followed by the arrival of lymphocytes and macrophages to control the spread of the pathogen. The final outcome of the *Salmonella* infection thus essentially depends on three factors: 1) the infective dose, 2) predisposing factor influencing the host, and 3) level of immunity (Ilyas *et al.*, 2017).

2.5. Context of the study

Recent studies (described in the literature reviewed above) on RpoE and Cpx systems have indicated the potential roles of these systems in pathogenesis, antibiotic resistance and biofilm formation. Microarray studies have also facilitated our understanding about the Cpx and RpoE-regulated genes. There are several genes that are predicted to be regulated by these systems and hence indicating their diverse role in bacterial adaptation to environmental stressors. There is growing appreciation regarding the complexity of these systems and their inter-ESR interaction. Besides, not much information is available on the role of these systems in *S.* Enteritidis, which are significant foodborne enteric pathogen and are known for its ability to form biofilm. Elucidating the role of RpoE and Cpx systems during biofilm formation in *S.* Enteritidis can help us to find alternate measures to eradicate the biofilm formation and manipulate the microbial genome for our own purposes. This thesis research was therefore designed to investigate the individual, as well as combined, role(s) of these systems in *S.* Enteritidis biofilm formation. I also plan to include the characterization of the Cpx regulon in the absence of *cpxR*, to further develop an understanding of CpxR-regulated genes that contribute to successful biofilm formation.

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BRIEF INTRODUCTION TO CHAPTER 3

Research in this chapter was performed to evaluate the role of the RpoE sigma factor and the Cpx two-component system in *Salmonella* biofilm formation. The chapter comprises of characterizing the Δ*rpoE*, Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains on basis of factors that influence biofilm formation followed by performing a detailed analysis of the ability of the mutants to form biofilm in comparison with the WT *S.* Enteritidis. Accordingly, two methods were employed to investigate biofilm formation by the mutants and WT strains: i) Crystal violet method ii) Flow cell method coupled with confocal microscopy. The results from this chapter provided preliminary evidence on the role of these systems in biofilm formation and also raised various unanswered questions involving the genetic changes behind the phenotypic differences observed due to the deletion of these regulators.

3. THE ROLE OF RPOE AND CPX SYSTEMS DURING *SALMONELLA* BIOFILM FORMATION

3.1.Abstract

The aim of this study was to investigate the role of RpoE and Cpx stress response systems during *S.* Enteritidis biofilm formation. Studies have highlighted the diverse physiological roles associated with the RpoE and Cpx systems which closely relates to explanations provided for biofilm formation. In this study, I investigated the involvement of RpoE and Cpx systems individually, as well as in combination, during *S.* Enteritidis biofilm formation. Using a flow cell system and confocal fluorescence microscopy, it was observed that *cpxR* knock-out strain (Δ*cpxR*) and double knock-out of both *rpoE* and *cpxR* (Δ*rpoE*/Δ*cpxR*) resulted in defective biofilm formation. The Δ*cpxR* and Δ*rpoE*/Δ*cpxR* significantly contributed to decreased biofilm biomass. The cells in the biofilm formed by Δ*cpxR* strain showed an unusual elongation/filamentation phenotype; however, the Δ*rpoE* strain was capable of forming biofilm phenotypically-similar to the (WT) *S.* Enteritidis. A positive correlation was seen between biofilm formation and auto-aggregation ability of these strains. The Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains showed weaker auto-aggregation; whereas, the Δ*rpoE* and WT strains showed stronger auto-aggregation ability. A significant ($p<0.05$) increase in motility was also seen by Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains indicating that CpxR might negatively regulate the expression of genes involved in motility. Taken together, these results highlight the important role of CpxR response regulator as well as the combined role of both RpoE and CpxR systems during biofilm formation in *S.* Enteritidis.

3.2.Introduction

Biofilms are recognized as a ubiquitous form of bacterial existence in the environment and have emerged as a primary survival strategy to withstand the harsh environmental conditions enforced by the natural or man-made activities. They are described as complex communities of microorganisms immobilized on a substratum which are embedded in a self-produced extracellular matrix (Sauer, 2003; Hall-stoodley & Stoodley, 2009). Biofilms thus provide a dynamic environment to the bacteria; however, the formation itself is a complex and highly-regulated process. Various bacterial surface components, primarily flagella, pili and fimbriae with roles in motility, adhesion and chemotaxis, are significant indicators of biofilm formation (Petrova & Sauer, 2012; Houdt & Michiels, 2010). Auto-aggregation, in which bacteria of same type form clumps, are known to be among the first steps of biofilm formation (Sorroche *et al.,* 2012; Kragh *et al.,* 2016). The ability of the bacteria to sense signals and shift from planktonic (free-living single bacteria) to biofilm lifestyle predominantly requires phenotypic and genetic changes termed "emergent properties" to proceed with the multi-step process of biofilm formation (Flemming *et al.,* 2016). In order to thrive in harsh environmental conditions, bacteria establish these emergent properties which includes, physical and social interactions, enhanced rate of genetic exchange, and increased antibiotic tolerance due to self-produced EPS to form biofilms on various abiotic and biotic surfaces. A number of studies have highlighted the ability of *S.* Enteritidis to form biofilm on various surfaces (Korber *et al.,* 1997), putatively providing resistance against stressors like temperature, antibiotics, biocides, etc. (Marin *et al.,* 2009; Scher *et al.,* 2005). *S.* Enteritidis represents a major cause of food-borne human gastroenteritis with significant impacts on public health sector world-wide (Lu *et al.,* 2003; Wisner *et al.,* 2010). Reports have highlighted the ability of *Salmonella* to adhere to various food surfaces, including tomatoes, almonds and cantaloupes, posing challenges to various food industries (Abrew *et al.,* 2018; Buck & Pathology, 2003). It has been reported that *S.* Enteritidis biofilms are linked with enhanced virulence and invasiveness, thus burdening the health care sector to treat infections (Guard- Petter *et al.,* 1996; Solano *et al.,* 2001).

Biofilms have severe impact in medical, food as well as marine industries that leads to substantial economic and health problems. One of the potential reasons could be the increased resistance provided by the penetration barrier that biofilms present to potential stress factors. Past report has linked the contribution of envelope stress response system in biofilm formation (Bury-Moné *et al.,* 2009). This chapter focuses on two of these envelope stress response systems for their

role in biofilm formation: RpoE sigma factor and Cpx two-component signal transduction system. The role of RpoE in biofilm formation has been demonstrated in Crohn's Disease-Associated Adherent-Invasive *E. coli* where decreased biofilm formation was observed in the absence of *rpoE*; whereas, enhanced biofilm development was seen in a *rpoE*-mutant strain of *S. pneumoniae* (Chassaing & Darfeuille-michaud, 2012; Churton *et al.,* 2016). Some of the genes involved in the processes significant to biofilm formation, such as motility, adhesion and chemotaxis, have been predicted to be governed by Cpx response system (Raivio, 2014). The role of the Cpx system in biofilm formation has been demonstrated in *E. coli* and recently in *Actinobacillus pleuropneumoniae* (Li *et al.*, 2018; Dorel *et al.,* 1999).

This study was designed to investigate the role of RpoE and Cpx system in *Salmonella* biofilm formation. Different experiments were employed to characterize the Δ*rpoE*, Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains based on their growth, motility and auto-aggregation properties. Their ability to form biofilms was demonstrated using a flow cell system wherein biofilms that developed by the different mutants and WT strains were non-destructively studied under confocal microscopy. It was accordingly found that although deletion of RpoE did not significantly influence biofilm formation, biofilms resulting from deletions of Δ*cpxR* and Δ*rpoE/*Δ*cpxR* were strikingly different from the WT *S*. Enteritidis, suggesting the importance of CpxR, the response regulator of Cpx system, during *S*. Enteritidis biofilm formation.

3.3. Materials and methods

3.3.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 3.3.1. *S.* Enteritidis ATCC 13076 strains were routinely grown at 37°C in Luria-Bertani (LB; Becton Dickinson, Cockeysville, MD) broth (pH 7.0) or tryptic soy broth (TSB; purchased from BBL (Becton Dickinson, Cockeysville, MD)). When required, the medium was supplemented with appropriate antibiotics at the following concentrations: 100 μg per ml ampicillin, 25 μg per ml chloramphenicol.

3.3.2. Growth assay

For analysis of growth kinetics, strains were grown overnight in LB medium. Overnight cultures were then diluted 100-fold into 100 ml of the same medium and incubated at room temperature (RT) with continuous shaking at 190 ± 5 rpm. Growth was monitored spectrophotometrically for 10 h by measuring optical density at 1 h intervals at OD_{600} nm. Data are the means of three replicates. The exponential growth rate was calculated based on the increase in OD_{600} over the optical density interval 0.025 to 0.3 for WT, and normalized to the growth rate of the corresponding Δ*cpxR*, Δ*rpoE* and Δ*cpxR* / Δ*rpoE* strains. The formula used to calculate growth rate (r) was r = (ln [OD2-OD1]) / (T2-T1) (Widdel, 2010).

3.3.3. Construction of Δ*cpxR*, Δ*rpoE* and Δ*cpxR*/Δ*rpoE S*. Enteritidis strains

All primers used and their purposes are listed in Table 2. Briefly, single (*rpoE, cpxR*) and double (*rpoE/cpxR)* deletion mutants were constructed by replacing corresponding genes with chloramphenicol cassette. The primers were designed to amplify the chloramphenicol cassette using pKD3 plasmid as a template, including 39-bp and 48-bp homologous extensions from the 5' and 3' ends of *rpoE* and *cpxR,* respectively. Using the λ red recombination system (Datsenko & Wanner, 2000), the chloramphenicol cassette targeting the respective gene for deletion was electroporated into recipient *S.* Enteritidis cells harbouring the *Red* helper plasmid pKD46 (containing the *red* genes encoded by Exo, Beta and Gam proteins). Colonies were recovered on LB medium with appropriate antibiotics. In order to excise the chloramphenicol cassette, the temperature-sensitive, *Flp* recombinase-expressing vector pCP20 was introduced via electroporation (Cherepanov & Wackernagel, 1995). pCP20 was accordingly cured from transformed strains by growing the clones at elevated temperature (42°C). All mutants were

verified by PCR amplification using primers listed in Table 3.3.2, followed by Sanger sequencing (Sanger & Nicklen, 1990).

Table 3. 1. Strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Strains		
S. Enteritidis		
ATCC 13076	Wild type; Cm^s, Amp^s	*Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC® 13076™)
Δ*rpoE*	ATCC 13076 with in-frame deletion of *rpoE*	This study
Δ*cpxR*	ATCC 13076 with in-frame deletion of *cpxR*	This study
Δ*rpoE*/Δ*cpxR*	Δ*rpoE* with in-frame deletion of *cpxR*	This study
Plasmids		
pKD3	Plasmid used as a template for construction of *Salmonella* mutant; Amp^r, Cm^r	James Imlay, School of Molecular and Cellular Biology, University of Illinois at Urbana-Champaign.
pKD46	λ Red helper plasmid, Amp^r	
pCP20	Plasmid containing recombinase flippase (*flp*), Amp^r, Cm^r	

Cm, chloramphenicol; Amp, ampicillin. Superscript r and s indicate resistance and sensitivity, respectively.

Table 3. 2. Oligonucleotides used in this study.

Gene amplified	Primers	Primer sequences (5'-3')	Purpose
Cm	*Salmonella* M rpoE-F[a]	ATG AGC GAG CAG TTA ACG GAC CAG GTC CTG GTT GAA CGG TGT AGG CTG GAG CTG CTT CG	Construction of Δ*rpoE*
Cm	*Salmonella* M rpoE-R[a]	TCA ACG CCT GAT AAG CGG TTG AAC TTT ATT ATC AAT AGC CAT ATG AAT ATC CTC CTT AG	Construction of Δ*rpoE*
Upstream of rpoE	RpoE mutant-F	GAC CTG TCT ACA ACA TGA CAA ACA	Verification of Δ*rpoE*
Downstream of rpoE	RpoE mutant-R	CGG ATC AGG TGA TAA CTC TCC CAG	Verification of Δ*rpoE*
Cm	*Salmonella* L cpxR-F[a]	ATT AGC GAC GCC TGA TGA CGT AAT TTC TGC CTC GGA GGT ACG TAA ACA TGT AGG CTG GAG CTG CTT CG	Construction of Δ*cpxR*
Cm	*Salmonella* L cpxR-R[a]	CCA GCG TCA ACC AGA AGA TGG CGA AGA TGC GCG CGG TTA AAC TTC CTA CAT ATG AAT ATC CTC CTT AG	Construction of Δ*cpxR*
Upstream of cpxR	cpxR mutant-F	CGC TTG CTC CCA AAA TCT TTT CTG	Verification of Δ*cpxR*
Downstream of cpxR	cpxR mutant-R	GTT GCT CTA TCA TCA ATC CCT GGC	Verification of Δ*cpxR*

[a] The underlined refer sequence homology with the chloramphenicol resistance gene (Cm) from plasmid pKD3.

3.3.4. Auto-aggregation assay

An auto-aggregation assay was performed based on the method previously described by Shanks *et al.* (2008), with minor modifications. Briefly, bacterial cultures were grown at 37°C for 24 h in LB medium. An aliquot (5 ml) of overnight culture was allowed to stand for 24 h at 25°C to evaluate auto-aggregation. The upper 1 ml of the overnight culture was carefully removed to measure its optical density (OD_{600}) (recorded as OD_{600} pre-vortex). The remaining culture in the test tube was then mixed by vortexing for 1 min to re-suspend the aggregated cells, and 1 ml of the suspension was removed and its OD_{600} was measured (recorded as OD_{600} post-vortex). The "percent aggregation" was calculated using the formula: 100 * (OD_{600} post-vortex - OD_{600} pre-vortex)/OD_{600} post-vortex. Meanwhile, microscopic images of WT and Δ*cpxR S*. Enteritidis after auto-aggregation were observed using phase contrast microscopy under oil immersion lens.

3.3.5. Motility assay

Bacterial swimming motility was assayed on LB plates containing 0.25% (w/v) agar, where bacteria can swim through the water channels inside the media. Using sterile toothpicks, single colonies from streaked LB plates of *S.* Enteritidis WT and mutant strains were stabbed into motility agar plate, from three independent colonies, and incubated for 24 h before visually assessing motility (dispersion distance in mm from point of inoculation) at 25°C.

3.3.6. Biofilm formation assays

3.3.6.1. Crystal violet biofilm assay

Biofilm formation in polystyrene microtiter plates (Greiner Bio-One, Germany) was assayed, as described by Čabarkapa *et al.* (2015) with minor modifications. Briefly, cells were grown in the wells of microtiter plates in 180 µL of TSB medium incubated for 48 h at 37°C. The medium was removed and the wells were washed with 250 µL of sterile distilled water. Subsequently, each well was stained with 250 µL of 0.5% (w/v) crystal violet for 10 min. After incubation at room temperature, the dye was removed and the wells were washed thoroughly with distilled water followed by air drying. Biofilm accumulation was quantified by solubilizing the bound crystal violet with 250 µL of 30% (v/v) acetic acid solution, and the absorbance was measured at 600 nm using an automated microtiter reader (Packard SpectraCount BS10000 absorbance microplate reader, 110 VAC). The biomass percentage was measured relative to WT using formula (mutant/WT*100).

3.3.6.2.Continuous flow biofilm culture assay

Biofilm formation in flow cells were assayed as previously described by Korber *et al.* (1994). Briefly, multi-channel flow cells [length (3.5 cm)* breadth (0.5 cm) * depth (0.5 cm)] were constructed using 5 mm sheets of polycarbonate plastic into which channels were milled and covered with #1 glass coverslips. The reactor system consisted a reservoir of sterile medium [10% (w/v) TSB] connected via silicone tubing to a bubble trap and subsequently to the flow cell followed by the effluent reservoir. The entire reactor system was sterilized by flushing 5.25% (w/v) sodium hypochlorite solution for a period of 15 min. followed by washing with sterile distilled water for 30 min. The medium was pumped through flow cells using a Watson-Marlow peristaltic pump (Model 202U; Watson-Marlow, Cornwall, UK). Each flow cell was inoculated with 0.5 ml of bacterial culture of an optical density 0.5 at 600 nm. The inoculum was retained in the flow cell for 30 min. at room temperature to facilitate cell adhesion of bacteria to the flow cell channel surfaces. The biofilms were grown at a nutrient laminar flow velocity of 0.2 cm sec^{-1} at room temperature for the duration of the assay. Biofilms were non-destructively analyzed using a Nikon C2 confocal scanning laser microscopy (CSLM; Nikon, Mississauga, ON, Canada) at 24 h, 48 h, 72 h and 96 h.

3.3.7. Confocal laser scanning microscopy (CLSM) and biofilm quantification

The biofilm was non-destructively analysed using CLSM which was used in conjunction with the BacLight™ LIVE/DEAD staining kit (Molecular Probes, Life Technologies, Burlington, ON) to quantify the distribution of bacterial biomass in biofilms. The BacLight™ LIVE/DEAD staining kit consists of SYTO 9 and propidium iodide (5 mM solutions in DMSO; Life Technologies, Carlsbad, CA, USA), and prepared according to the manufacturer's guidelines (SYTO 9 and propidium iodide manuals, 2011). Then, a 2 µl aliquot of the dye working solution was added to 1 mL 1X Phosphate Buffered Saline (PBS), pH 7.4, vortexed, and directly added into the flow cell using 1-cc syringe. Biofilms were examined using a Nikon C2 fluorescence scanning confocal laser system (CLSM; Nikon, Mississauga, ON, Canada). Dual-channel images, corresponding to fluorescence emission in the green (excitation/emission 488/522 nm) (SYTO 9) and red (excitation/emission 535/617 nm) (propidium iodide) wavelengths, were acquired in the horizontal (*xy*) and vertical (*xz*) planes. Images were obtained using an 60X Plan Apo VC (N.A. 1.4, Nikon) objective lens. Biomass estimation was carried out by collecting optical thin sections (OTS) using a *z*-step increment of 0.9 µm from the attachment surface (i.e. 0.9, 3.6, 6.3, 9 and 11.7

μm) at five randomly-chosen locations, with the NIS Element Confocal Microscope Imaging Software (version 4.10) used to quantify biomass abundance in each OTS. The biomass percentage at each OTS was measured relative to WT using formula (mutant/WT*100). The mean total biomass is the result of three independent experiments. Biofilm thickness was measured in micrometers (μm) using a computer-controlled, motorized z-axis stepper motor (Korber *et al*., 1994). Fifteen random fields were assessed for each biofilm with five separate thickness values obtained per field (n = 75). These values were averaged to obtain the thickness data for each biofilm.

3.3.8. Experimental replications and statistical analysis

All experimental data represent the arithmetic mean of at least three independent experiments. Biofilm thicknesses were analyzed using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC), and the PROC t-test was used to test for significant ($P < 0.05$) differences.

3.4.Results

3.4.1. Construction of *S*. Enteritidis Δ*rpoE*, Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains

In order to investigate the individual and combined roles of the extracytoplasmic stress response regulators, the RpoE and CpxR systems were disabled by the in-frame deletion of *rpoE* and *cpxR* (response regulator) genes, respectively. Using the λ red mutagenesis (Datsenko & Wanner, 2000), the coding region of *rpoE* and *cpxR* was replaced with a chloramphenicol resistance construct, as described above, to generate Δ*rpoE,* Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains. Further, the chloramphenicol cassette was removed with the help of pCP20 plasmid to generate final "insertion-free" mutants. All the three mutants were verified using PCR band analysis, as well as Sanger sequencing. The analyses revealed the complete deletion of gene of interest, with an ~70 bp 'scar' of plasmid pKD3 remaining in *S*. Enteritidis Δ*rpoE*, *cpxR* and Δ*rpoE*/Δ*cpxR* strains. The colony morphology of the mutants was similar to that of WT *S*. Enteritidis. Gel images confirming the mutants by analyzing the band size of the PCR product are provided in Figure 3.4.1.

3.4.2. Extracytoplasmic stress response regulators, RpoE and CpxR, differentially-affect growth rate, motility and auto-aggregation in *S*. Enteritidis

Growth curve analysis revealed no significant differences between WT and Δ*cpxR S*. Enteritidis strains when grown aerobically in LB broth at RT. The growth rate due to deletion of *cpxR* gene was not affected relative to WT strain. However, the Δ*rpoE* strain showed an extended lag phase with lower final optical density relative to WT and Δ*cpxR* strain (Figure 3.4.2). Deletion of *rpoE* gene reduced the exponential growth rate by 20% [from 0.6 (WT) to 0.48 (Δ*rpoE*) h^{-1}]. A previous study in *Streptococcus mutans* highlighted the essential role of *rpoE* for optimum growth and indicated that genetic and metabolic changes in an Δ*rpoE* caused impaired growth (Xue *et al.,* 2010). Furthermore, the growth deficiency was also reported during exponential phase in the Δ*rpoE E. coli* (Vidovic *et al.,* 2018). In my thesis work, it was noted that a severe growth deficiency was seen in case of the Δ*rpoE*/Δ*cpxR* strain. Deletion of both the genes had much stronger impact on the fitness, reducing the growth rate by 98%. These findings indicate that although *rpoE* is required for optimal growth, both RpoE and CpxR have a synergistic effect on the growth rate of *S*. Enteritidis.

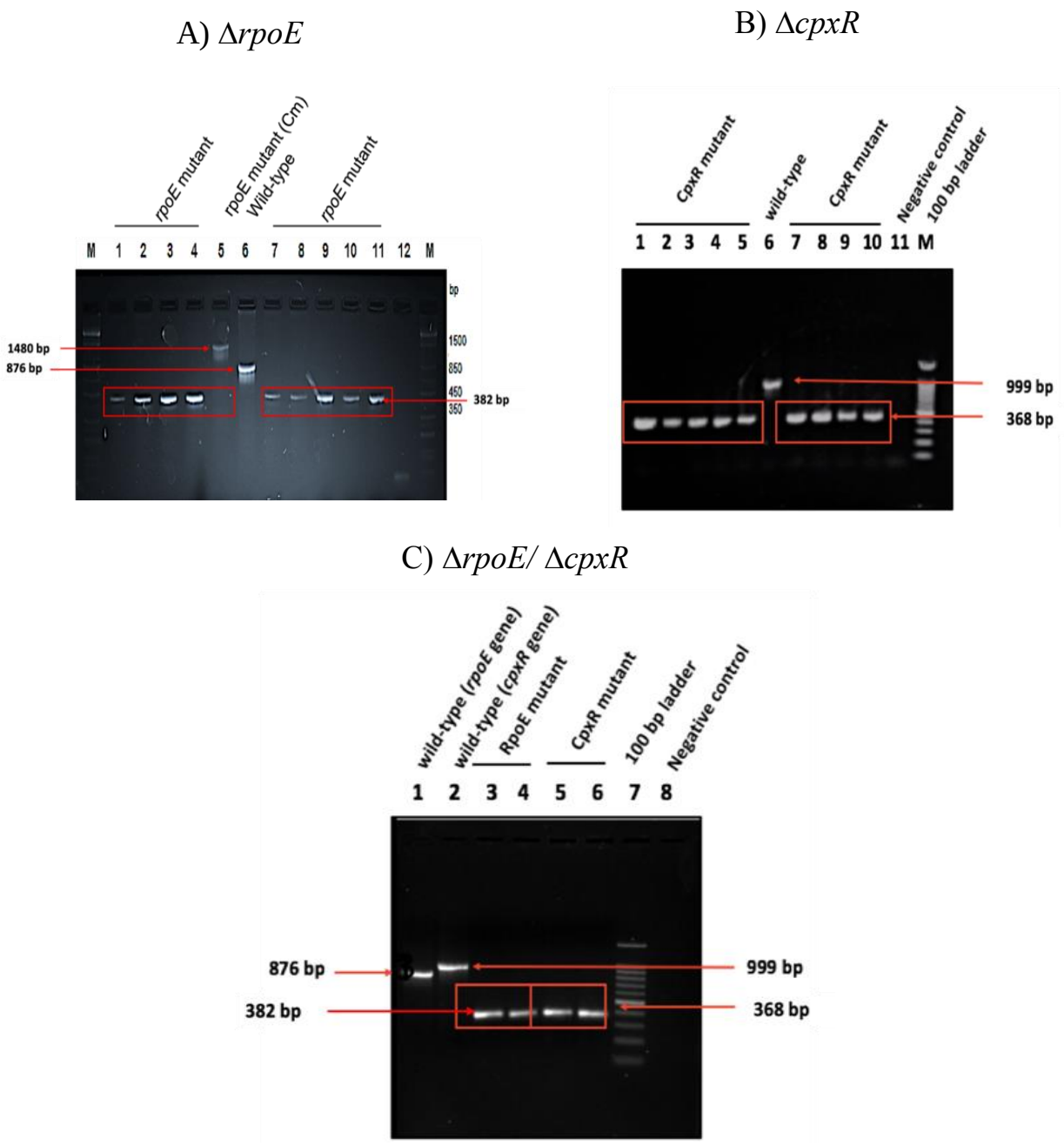


Figure 3. 1. PCR verification of Δ*rpoE* , Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains of *S*. Enteritidis. PCR verification was performed as described in Section 3.3.3, using oligonucleotide primers listed in Table 2. All products were run on 1% agarose gels and the DNA fragments stained using 0.5 µg/ml EtBr visualized under UV light with a 100 bp DNA ladder for reference. A) The expected amplicon sizes are Δ*rpoE*: 382 bp; Δ*rpoE* (Cm): 1480 bp and WT: 876 bp, B) The expected amplicon size for Δ*cpxR*: 368 bp and WT: 999 bp, C) The expected amplicon size for Δ*rpoE* and Δ*cpxR*: 382 bp and 368 bp, respectively.

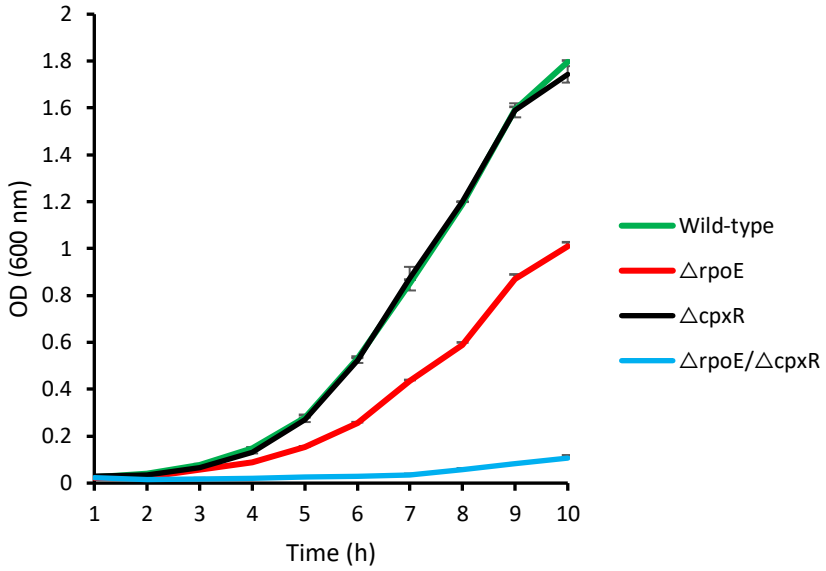


Figure 3. 2. Effect of deletion of the *rpoE*, cpxR and *rpoE/cpxR* genes on the growth of *S.* Enteritidis in LB at 22°C±3. Data shown represents the average of at least three independent experiments. A significant decrease in growth was seen in Δ*rpoE* as well as Δ*rpoE*/Δ*cpxR* strain when compared with the wild-type and Δ*cpxR* strains ($p < 0.001$). The error bars represent the standard deviation determined by t-test on comparison with wild-type and Δ*cpxR* strains.

Furthermore, we quantified the auto-aggregation behaviour of planktonic WT and mutant *S*. Enteritidis strains. Auto-aggregation, based on adhesive interaction ability of the bacteria, may play a crucial role in the initial steps of biofilm formation (Xue *et al.*, 2010). The following criteria were used to classify the auto-aggregation observed in WT and the mutant strains: strong auto-aggregation (≥ 70%), moderate auto-aggregation (≥ 30%), weak auto-aggregation (< 30%). As shown in Figure 3.4.3-A and 3.4.3-B, the *S*. Enteritidis WT strain displayed a strong auto-aggregation (~80%) tendency compared to the all mutant strains. A much weaker auto-aggregation trend was shown by Δ*cpxR* (~20%) which remained at a constant turbidity. The Δ*rpoE* (~60%) and Δ*rpoE*/Δ*cpxR* (~38%) strains showed comparatively moderate auto-aggregation relative to Δ*cpxR*. Auto-aggregation ability of WT and Δ*cpxR* strains was also evaluated microscopically to approximate aggregate size and their distribution in the sample (Figure 3.4.3-C). WT *S*. Enteritidis cells formed tightly-packed aggregates (Figure 3.4.3-C; indicated by red arrows); whereas, Δ*cpxR* cells remained as single cells in the suspension. Overall, these results suggests that the reduced ability of the Δ*cpxR* to auto-aggregate may be because of the down-regulation of genes involved in auto-aggregation which are directly or indirectly regulated by CpxR.

I also quantified the swimming motility of the WT and the mutant *S*. Enteritidis strains at 22±3°C in LB using conventional soft agar (media containing 0.25% agar). The motility of Δ*rpoE* was not affected relative to the WT motility. However, an increase in motility was observed in the Δ*cpxR* (the diameter of motility zone was more than twice of WT) compared to the WT strain. From the literature, it is known that the Cpx system negatively-regulates the expression of the *tsr* and *motABcheAW* operons, involved in chemotaxis and motility (De Wulf *et al.*, 2002). Thus, the in-frame deletion of *cpxR* resulted in increased motility by virtue of the absence of negative regulatory control. In the Δ*rpoE*/Δ*cpxR*, motility was reduced compared to Δ*cpxR*; however, it was significantly increased (the diameter of motility zone was roughly twice of WT) compared to the WT. As seen in Figure 3.4.4-A and 3.4.4-B, motility was slightly compromised in the Δ*rpoE*, whereas the Δ*cpxR* showed increased motility. Thus, it is possible that the in-frame deletion of both *rpoE* and *cpxR* may have reduced the effect of motility enhancement in Δ*rpoE*/Δ*cpxR*.

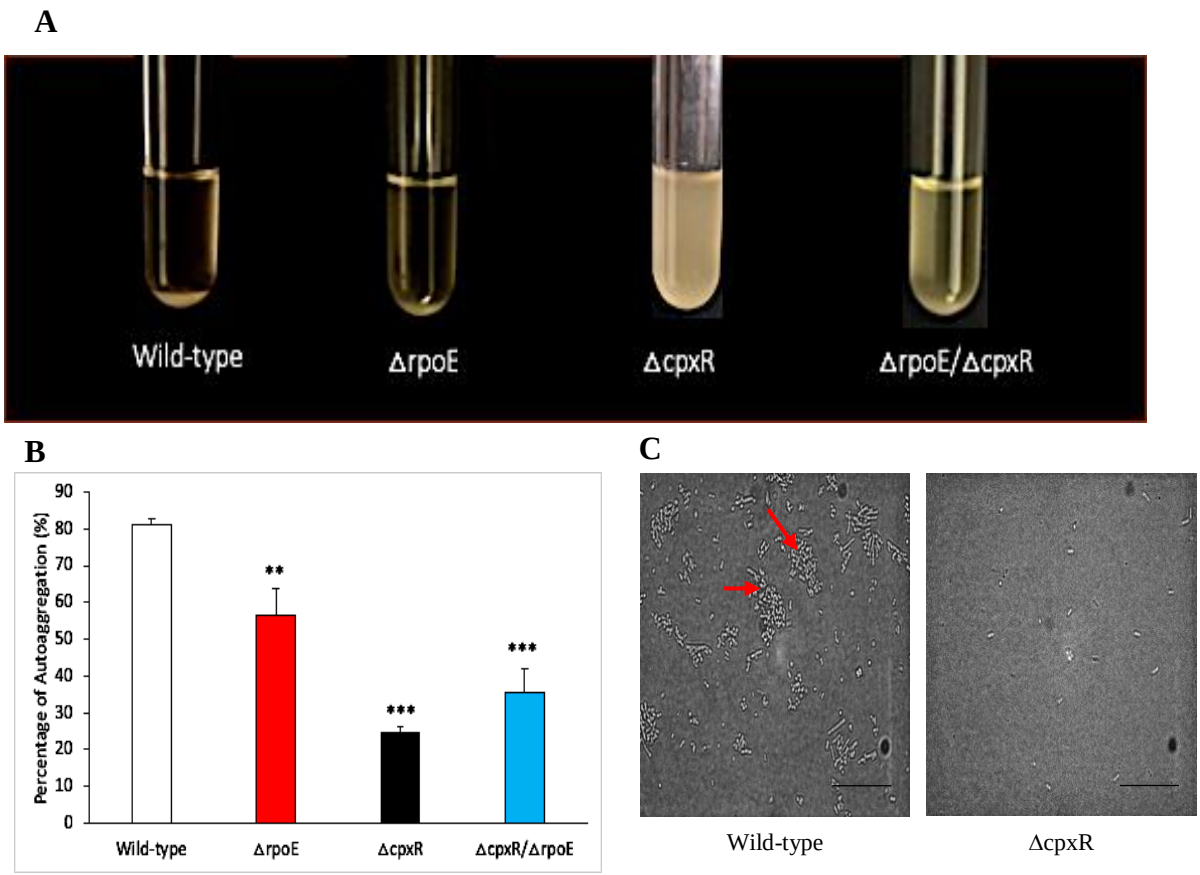
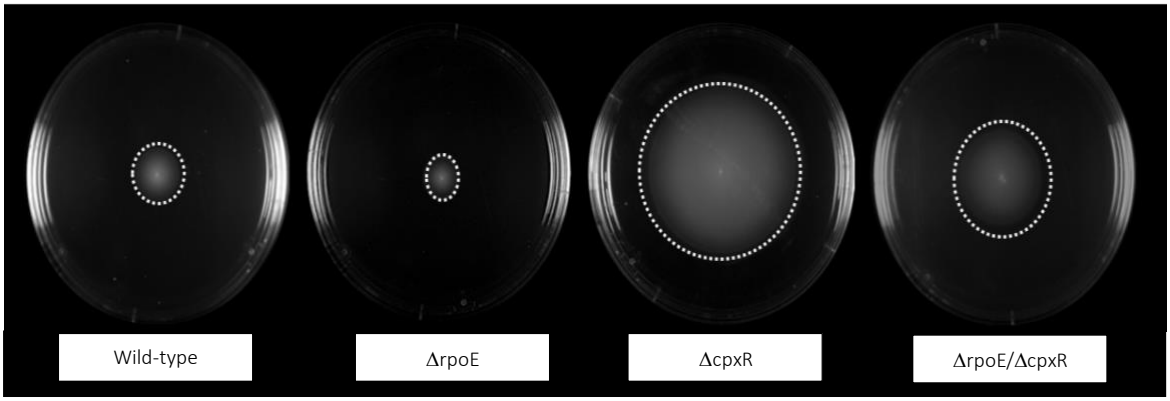


Figure 3. 3. Auto-aggregation Assay. A) Macroscopic analysis of auto-aggregation in wild-type *S.* Enteritidis, which was highly-aggregative, compared to the Δ*cpxR*, which remained turbid (non-aggregative) illustrates the strong effect of the CpxR mutation. Δ*rpoE* and Δ*rpoE*/Δ*cpxR* strains showed moderate auto-aggregation. B) Quantitative illustration of sedimentation-based auto-aggregation assay of wild-type and mutant strains of *S.* Enteritidis. Each data point represents the average of at least three independent experiments. The error bars represent the standard deviation determined by T-test on comparison with wild-type (*P < 0.05, ** P < 0.01, ***P < 0.001). C) Microscopic analysis of cells from the auto-aggregation assay using phase contrast microscopy. Red arrows depicts cell aggregates. Scale = 20 μm

A



B

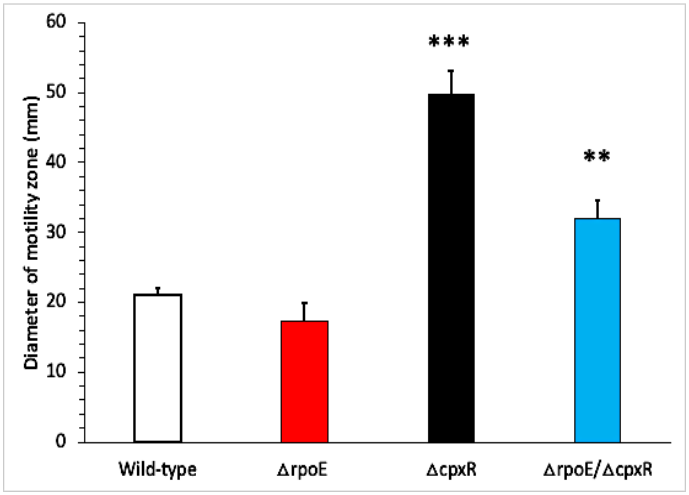


Figure 3. 4. Motility Assay. A) Swimming motility of wild-type and mutant *S.* Enteritidis strains in motility agar (0.25% agar and LB) for a growth period of 24 h at 22°±3. B) Quantification of swim ring diameter of wild-type and mutant strains. Each data point represents the average of at least three independent experiments. The error bars represent the standard deviation determined by t-test with comparison with wild-type (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).

3.4.3. CpxR has a profound effect on biofilm formation

To study the role of RpoE and Cpx regulatory systems in biofilm formation, two methods of analysis were employed: the CV assay of biofilms formed in multi-well polystyrene plates and CLSM analysis of biofilms grown in continuous flow slide culture. The CV assay was used to compare the ability of the WT and mutants to form biofilms. On comparing the percent biomass of WT *S.* Enteritidis to the Δ*rpoE*, the Δ*cpxR* and the Δ*rpoE*/Δ*cpxR* strains, a decrease of 78.5%, 87.72% and 86.2% was observed, respectively, thus indicating that the mutants were relatively-defective in biofilm formation (Figure 3.4.5-A and 3.4.5-B). Lack of biofilm development in the case of these mutants could be due to poor production of exopolysaccharides, lack of stimuli for adhesion and growth, or the failure to express genes responsible for adhesion to the surface. The above results suggest possible involvement of these pathways in the biofilm formation in *S. Enteritidis.* To validate the primary screening results of biofilm formed by WT and the mutant strains, CLSM approach was used to visualize the topography and baseline biofilm structure grown under continuous-flow, fully-hydrated conditions. Figure 3.4.6 shows representative CLSM micrographs of WT, Δ*rpoE*, Δ*cpxR* and Δ*rpoE*/Δ*cpxR* at 24, 48, 72 and 96 h of biofilm development. Key differences were seen in the architecture of WT and the mutant *S. Enteritidis* strains. As shown in Figure 3.4.6, there was significant increase in the biofilm biomass, along with a reduction in porosity, observed in both the WT and Δ*rpoE* biofilms.

Contrary to the results obtained from crystal violet, the Δ*rpoE* strain was able to form the biofilm similar to those formed by WT strain. One reason for this could be due to difference in the ability of the Δ*rpoE* to adhere to polystyrene and polycarbonate surfaces. Alternatively, the stimulatory effect of continuous flow of sterile growth medium, compared to recirculation of used growth medium with accumulating waste products in the wells of the polystyrene plates, could explain these differences. An unusual morphological change was seen in the biofilms formed by the Δ*cpxR* strain. Microscopic analysis demonstrated highly-elongated cells (filamentation) of Δ*cpxR* strain under biofilm condition in continuous flow slide culture (Figure 3.4.6; Δ*cpxR*). The unusual elongated cells were floating and weakly anchored to the surface which were clustered in packs and distributed throughout the flow channels. Previously, cell elongation was reported in *Pseudomonas aeruginosa* under anaerobic growth conditions and it was attributed to abnormally altered cell division. This unusual filamentous structure might indicate the pleomorphic behavior

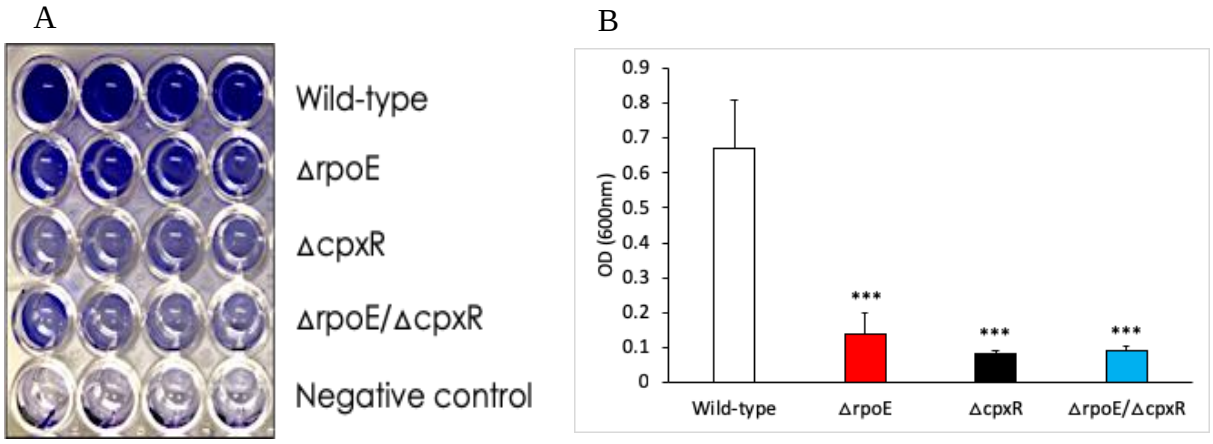


Figure 3. 5. A) Biofilm formation in the wells of 96-well polystyrene microtiter plates containing TSB and maintained at 37°C. Crystal violet was used to stain the biofilm in each well. B) Quantitative determination of biofilm formation. Each data point represents the average of at least four independent experiments. The error bars represent the standard deviation determined by T-test on comparison with wild-type (*P < 0.05, ** P < 0.01, ***P < 0.001)

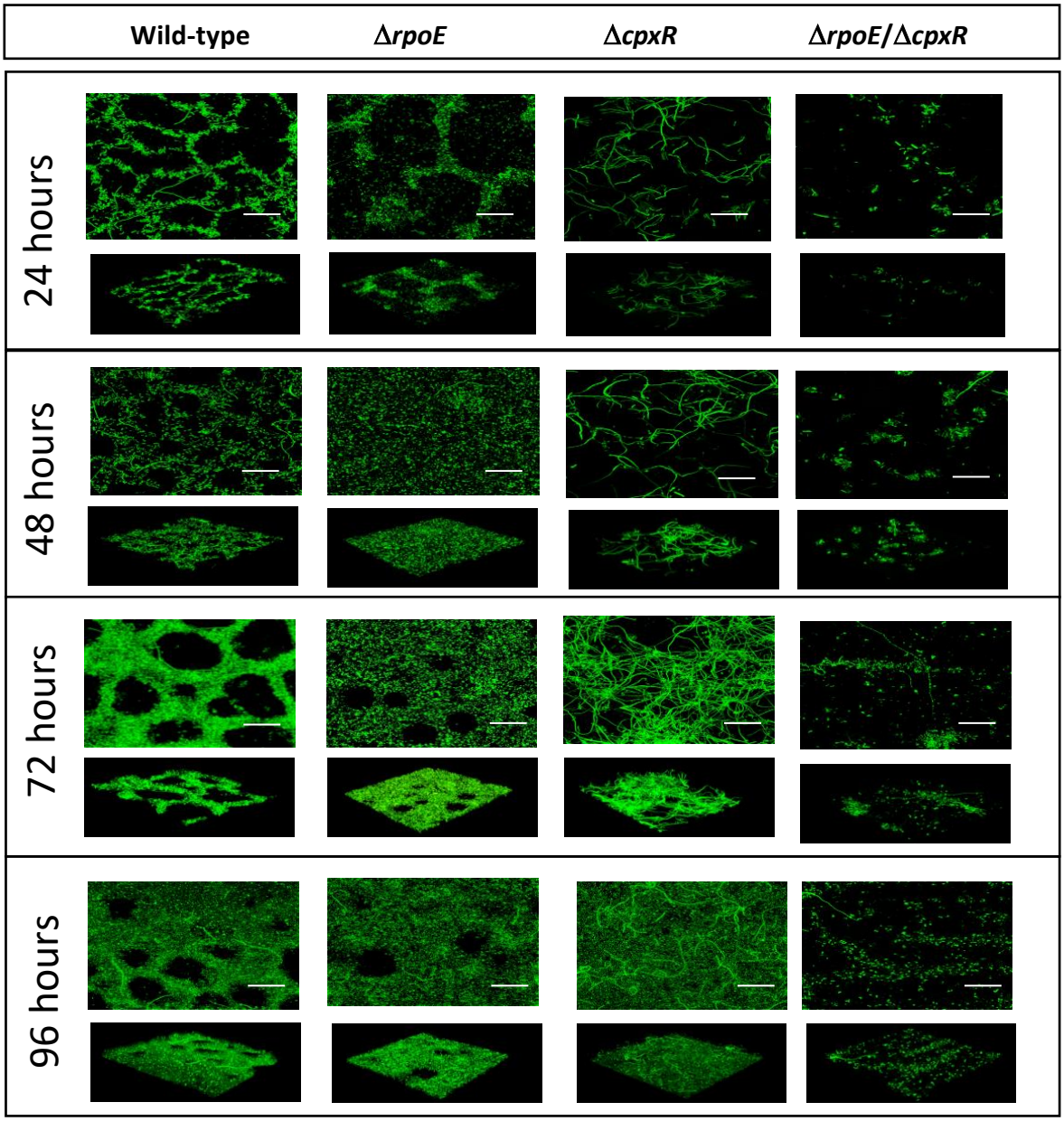
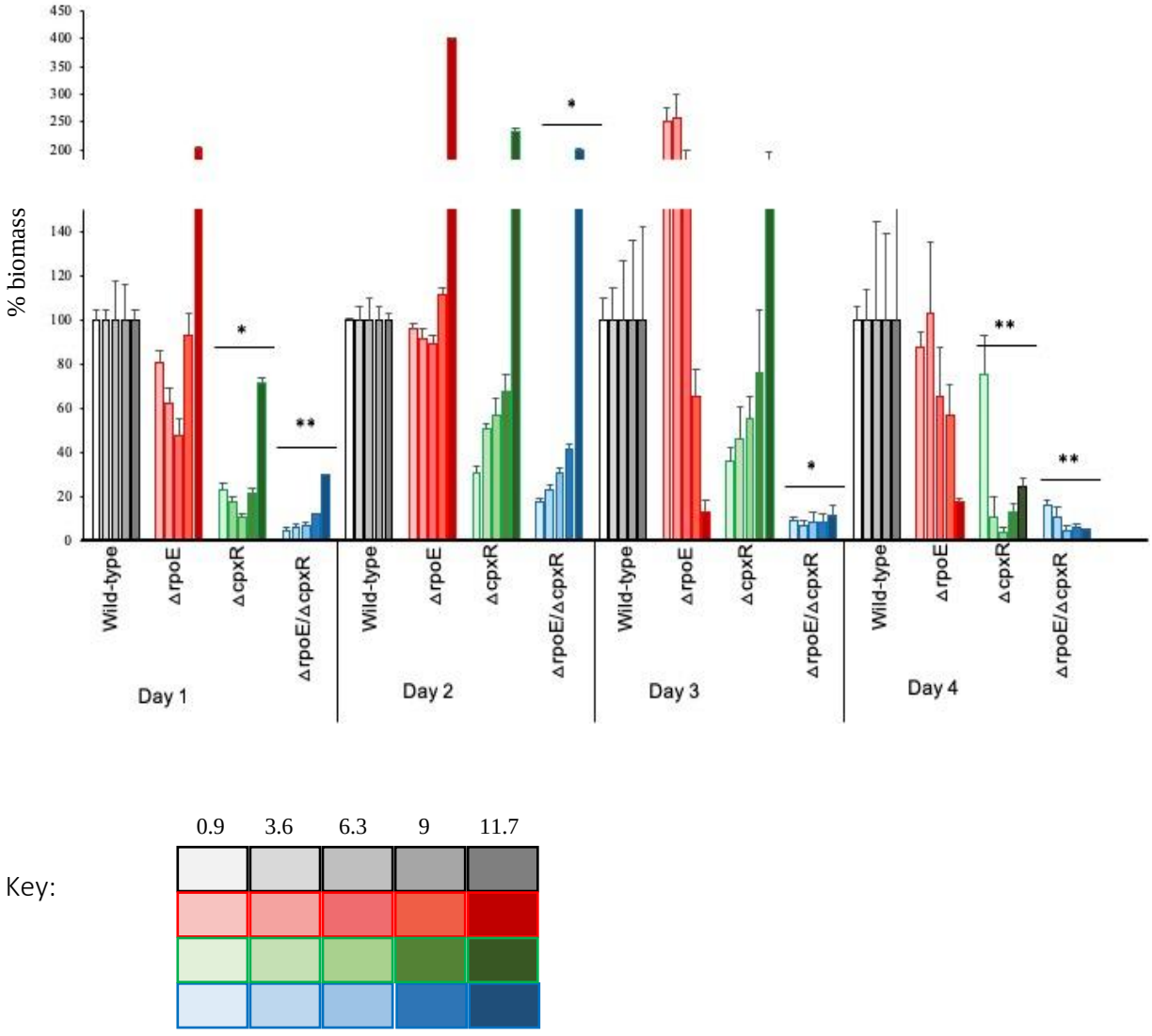


Figure 3. 6. Comparison of biofilm formation by wild-type, Δ*rpoE*, Δ*cpxR* and Δ*rpoE*/Δ*cpxR* strains over a 96 h time-course. Bacteria formed in the flow cells were stained with BacLight Live/Dead stain and observed under 60X magnification using a Nikon C2 confocal scanning microscope. A series of images were acquired at 0.9 μm interval along the Z-section and a three-dimensional (3D) biofilm architectures were constructed using NIS Elements Confocal Microscope Imaging Software (version 4.10). A top-view and the lateral (side) view are displayed for each biofilm. Scale bar = 20 μm

of Δ*cpxR*, which could possibly be the result of the development of nutrient gradients within the biofilm, causing the bacteria to undergo adaptation for their survival. Defects in cell division could also result in changes to the cellular morphology under biofilm conditions, possibly via a stress response triggered by DNA damage or just as a consequence of the mutation. The abundance of filamentous cell clusters increased gradually after 24 h, and hence a change in the thickness was observed at 48 h, confirming the change in the length of filamentous cells and the extent of vertical biofilm growth (thickness). At 72 h, these growing networks of filamentous cells started interconnecting with neighbouring clusters, and by 96 h there was no predominant filamentous cell material remaining, with a decrease in the number of filaments and corresponding increase in the number of small attached cells resembling the WT morphology. Quantitative analysis of biofilm biomass (Figure 3.4.7) and thickness (Figure 3.4.8) was calculated from CLSM images using NIS Elements Imaging Software to quantify the morphological and structural characteristics. The mean thickness of the biofilm formed by Δ*rpoE*/Δ*cpxR* (~8 μm) was significantly lower compared to wild-type (18 μm), Δ*rpoE* (19 μm) and Δ*cpxR* (16 μm) strains after day one (24 h). However, the biomass formed by Δ*cpxR* as well as Δ*rpoE*/Δ*cpxR* was significantly less than WT and Δ*rpoE S. Enteritidis* strains. By day two (48 h) and day three (72 h), there was a sudden increase in the thickness of the biofilm formed by Δ*cpxR* as the filaments increased in number (37 μm); whereas, no significant increase in the thicknesses of WT, Δ*rpoE* and Δ*rpoE*/Δ*cpxR* were observed. By day four (96 h), the thickness (27 μm), as well as the relative abundance of biofilm biomass, of Δ*cpxR* biofilm decreased as the number of filaments decreased gradually and the biofilm appeared flat and homogeneous. A constant decrease in biofilm biomass was seen from initial day one levels over the remaining incubation period for the Δ*rpoE*/*ΔcpxR* strain when compared to WT, Δ*rpoE* and Δ*cpxR* . Thus, deletion of both *rpoE* and *cpxR* in Δ*rpoE*/Δ*cpxR* strains clearly resulted in significantly impaired biofilm formation. Taken together, these data suggest an essential role of CpxR regulatory protein for classic, or WT, biofilm formation.



Key:

Figure 3. 7. Comparison of the total abundance of biofilm biomass of wild-type, Δ*rpoE*, Δ*cpxR* and Δ*rpoE/*Δ*cpxR* strains of *S.* Enteritidis over a 4-day developmental time-course. The total biomass at each time interval and at each optical thin section (OTS) depth was determined at five randomly-chosen locations for each of the three biological replications. The 0.9 μm OTS represents the biofilm-substratum interface. The percentage biomass was calculated relative to wild-type, using the formula mutant/wild-type *100. The Color Key indicates the percentage total biomass, relative to the wild-type control biofilm, at 0.9, 3.6, 6.3, 9 and 11.7 μm OTS depths, respectively. The error bars represent the standard deviation determined by t-test on comparison with wild-type (*P < 0.05, ** P < 0.01, ***P < 0.001).

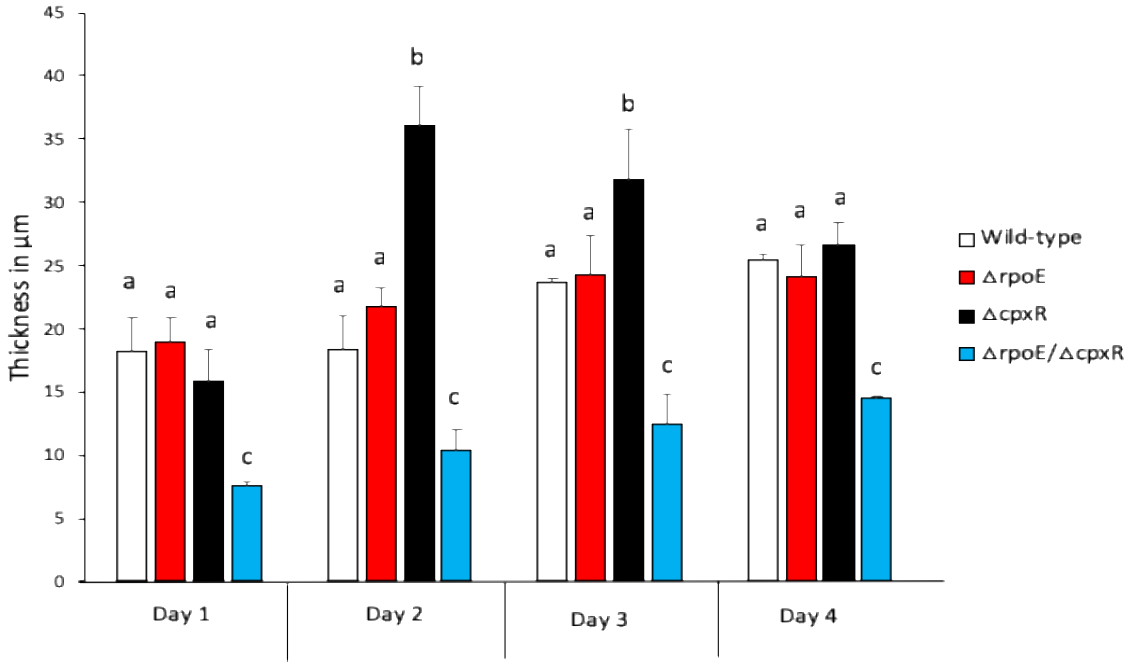


Figure 3. 8. Comparison of biofilm thickness of wild-type, Δ*rpoE*, Δ*cpxR* and Δ*rpoE*/Δ*cpxR* of *S.* Enteritidis. The thickness measurements at each time interval are the average of 225 individual thickness measurements made at random locations from three independent (biologically-replicated) biofilms (n=75 for each biofilm). The error bars represent the standard deviation determined by t-test on comparison with wild-type. Means that were found to be significantly different ($p < 0.05$) by t-test are indicated by different letters.

3.5. Discussion

A growing number of studies indicates the role of RpoE and Cpx envelope stress response systems during biofilm formation and pathogenesis in numerous Gram negative bacteria [see Review by Rowley *et al.,* (2006)]. Besides, recent studies characterizing the RpoE and Cpx regulon proposes the role of these system in modulating the expression of genes involved in motility and adhesion which are crucial steps in biofilm formation (Gangaiah *et al.,* 2013; Labandeira-Rey *et al.,* 2010; Bury-Moné *et al.,* 2009; Raivio *et al.,* 2013; Xue *et al.,* 2012). The findings from the present study provides several lines of evidence strongly supporting the role of CpxR, a response regulator in Cpx two-component system, in biofilm formation by *S.* Enteritidis. Moreover, results from this study also directs us to think whether CpxR modulates the activity of RpoE along with other extracytoplasmic stress response systems whose roles are essential for bacteria to survive in specific environmental condition.

My thesis studies began with first physiologically-characterizing the Δ*rpoE,* Δ*cpxR* and Δ*rpoE/*Δ*cpxR* strains on the basis of their growth, motility and auto-aggregation ability followed by investigating their role in biofilm formation. In the present study, I observed that the absence of *cpxR* did not affect the growth rate; however, the absence of *rpoE* resulted in slower growth of mutant strain. Notably, the deletion of both *rpoE* and *cpxR* severely affected the growth of mutant in LB media at RT, thus indicating that the overlapping genes essential for the growth might be affected by both these regulators. Moreover, a significant increase in motility was observed in absence of *cpxR*, suggesting that motility genes are negatively-regulated by the CpxR response regulator whereas no significant difference was observed on deletion of *rpoE*. In case of absence of both *rpoE* and *cpxR* genes, there was an increase in motility; however, the diameter of dispersion was less compared to Δ*cpxR* strain. Furthermore, the ability to auto-aggregate was significantly weaker in case of Δ*cpxR* compared to WT strain. Moderate auto-aggregation ability was observed in Δ*rpoE* and Δ*rpoE/*Δ*cpxR* strains compared to WT strain. Studies have demonstrated that although flagella contributed to motility may play a role in the architecture of the biofilm, but are not required for initial adhesion. In fact, high motility might provide insufficient time to adhere to the surface to initiate irreversible attachment, thus inhibiting the biofilm formation (Bridier *et al.,* 2011). Korber *et al.* 1989 demonstrated in *P. fluorescens* that motility was correlated with increased attachment. Biofilm formation was similarly enhanced (Korber *et al.,* 1989; Korber *et al.,* 1990).

To summarize, some of the key results from the above experiments in association with the characteristics essential for biofilm formation include:

i) Deletion of *cpxR* did not affect the growth of the *S.* Enteritidis

ii) Motility significantly increased whereas the ability to auto-aggregate significantly decreased by deletion of *cpxR*

These complementary observations indicate investigations in the role of these systems and specifically CpxR in biofilm formation may be fruitful. In order to achieve our first goal of investigating the role of RpoE and Cpx regulatory systems in biofilm formation, Δ*rpoE,* Δ*cpxR* and both Δ*rpoE/*Δ*cpxR* strains were subjected to biofilm formation and were compared with WT classical biofilms. On one hand, I found that deletion of *rpoE* gene did not significantly affect the ability of *S.* Enteritidis to form biofilm as the morphology and the architecture of the biofilm was similar to that of WT; however, the Δ*rpoE* strain had a reduced ability to adhere to the microtiter plate. The exact reason is unknown, but the difference could be explained by the difference in the surface material. Microtiter plates are made up of polystyrene and the flow cells are made from polycarbonate sheets. Other reasons as cited above; 1) continuous flow of sterile, fresh nutrient medium, 2) depletion of nutrients and accumulation of wastes in polycarbonate wells.

On the other hand, deletion of *cpxR* gene not only altered the biofilm architecture but also the morphology of cells in the biofilm. The phenotypic elongation of cells into filaments was reported throughout the biofilm matrix with decreased biofilm biomass. Filamentation can occur due to several reasons, especially when exposed to stress (Fredborg *et al.,* 2015). It is known that several cellular stressors could be produced inside biofilm (Miranda *et al.,* 2010) and absence of *cpxR* gene in Δ*cpxR* strain could contribute to the inability of cells to respond to these stresses. Filamentous morphology could represent a strategy of the bacterial cells to resist stress as filamentation could offer several advantages including survival within host tissue (Justice *et al.,* 2006; Lecuyer *et al.,* 2013) as well as decreased susceptibility to certain antimicrobial agents (Justice *et al.,* 2008). However, switching to an elongated cell phenotype may not be sufficient to overcome environmental stress (Fredborg *et al.,* 2015). An alternate reason for filamentation could be the linked to genes having roles in cell division or peptidoglycan synthesis which confers shape to the bacterial cell becoming directly regulated by CpxR, which would be down-regulated or unexpressed in absence of CpxR. However, these are just few speculations and probably the characterization of the Cpx regulon (Chapter 4) in absence of *cpxR* under biofilm conditions might

provide better understanding of the role of CpxR in cell elongation phenomenon. Interestingly, after a period of 72 h, the number of elongated cells decreased concurrent with an increase in the number of small attached cells, thus returning the biofilm to a WT cell phenotype and overall architecture. Thus, the morphological defects associated due to deletion of *cpxR* gene were unstable. Reasons for this could be that the Δ*cpxR* might have developed suppressor mutations which could have reversed the morphological abnormalities. Recent study in *E. coli* demonstrated that interruption in O-antigen biogenesis, a major outer membrane component, indirectly impaired peptidoglycan (PG) synthesis thus resulting in elongation of *E. coli* cells (Jorgenson & Young, 2016). In this study, a reversal of the phenotypic changes was observed with cells reverting back to their original phenotype. This was because the mutants having deletion in *wzxB*, an O-antigen flippase, readily-developed suppressor mutations. It would be interesting to investigate whether CpxR directly plays a regulatory role of the genes required for O-antigen synthesis. If so, then which are those genes and how do they contribute to O-antigen synthesis? In addition to this, deletion of both *rpoE* and *cpxR* genes severely-impacted biofilm formation, resulting in very little to no biofilm formation by the Δ*rpoE*/Δ*cpxR* strain. Several possibilities include the following:

- One possibility is that CpxR might function more as a modulator for other ESRs, especially RpoE, rather than as a stand-alone regulator in biofilm formation. This possibility is based on the biofilm formation observed by all three mutants in comparison with WT. As observed, RpoE did not significantly contribute to biofilm formation which could be due to presence of CpxR of the Cpx system, which acts as a modulator for other ESRs to cover the functions of RpoE essential for biofilm formation. However, in the Δ*rpoE*/Δ*cpxR* strain due to deletion of *cpxR*, there was no modulator to alter the expression of other ESRs to counterbalance the effects of deleting both *rpoE* and *cpxR*, and thus the Δ*rpoE*/Δ*cpxR* strain lacked the ability to form biofilm.
- The other reason could be linked to the defect in cell growth, as observed in planktonic growth studies; however, even after 96 h, there was no significant biofilm biomass in Δ*rpoE*/Δ*cpxR* strain.
- Lastly, although no phenotypic changes were observed in biofilms formed by the Δ*rpoE* strain, there could be genetic alteration which did not significantly affect the phenotype. However, deletion of both *rpoE* and *cpxR* hints at the regulation of overlapping genes by

RpoE and CpxR, which together could have made the situation worse as both regulators resulted in very low or unexpressed genes essential for biofilm formation.

These studies on RpoE and Cpx stress signaling systems have provided new insights in their role in biofilm formation and the phenotypic changes linked with the deletion of their genes. Given the high specialization of each system, and the interconnected safety provided by these systems, there is growing appreciation of the complexity of these systems. The above studies thus put forth some new questions of the role of these systems individually, as well as in combination, during *Salmonella* biofilm formation.

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BRIEF INTRODUCTION TO CHAPTER 4

This chapter is an extension of the previous chapter that examined the individual and synergistic roles of RpoE and CpxR stress response systems in *S.* Enteritidis biofilm formation. With an intention to further investigate the genes regulated by RpoE/CpxR and CpxR, I grew biofilms formed by wild-type, Δ*cpxR* and Δ*rpoE/*Δ*cpxR* strains, respectively, in order to collect nucleic acids for RNA sequencing. However, due to the low biofilm biomass formed by *rpoE/cpxR* knock-out mutant strain, I focused on *cpxR* knock-out mutant strain (in comparison with the wild-type) to determine the underlying genes regulated by CpxR which were responsible for decreased biofilm biomass and the phenotypic shift from short rod-shaped cells to elongated filaments in the biofilm matrix. The following chapter thus provides a genetic explanation for the various phenotypic changes that were observed due to the absence of *cpxR* in both planktonic and biofilm cell cultures.

4. TRANSCRIPTION CONTROL OF CPX STRESS-SIGNALING RESPONSE SYSTEM DURING *SALMONELLA* BIOFILM FORMATION

4.1. Abstract

Several stress response systems are predicted to control the genetic signals that modulate the biofilm development at every stage including attachment, maturation and dispersion. The previous chapter concluded that the response regulator CpxR of the two-component Cpx system significantly contributed to biofilm formation in *S.* Enteritidis by decreasing the abundance of biofilm biomass formed, and by exhibiting an unusual filamentous phenotype of the cells in the biofilm network. In the present study, Comparative transcriptomic approach was employed to characterize the changes in the transcriptomic profiles of Δ*cpxR* and wild-type (WT) *S.* Enteritidis in planktonic (free-floating single bacteria) and biofilm-associated cell cultures. Transcriptomic analysis revealed three groups based on the gene expression profiles in the Δ*cpxR* strain: i) genes unexpressed in Δ*cpxR* strain (may have some background level of expression but below the detection level), ii) genes significantly up- or down-regulated, or iii) genes that undergo little or no change compared to WT. A set of 223 genes were identified (most of which were involved in biofilm formation and pathogenesis) which were not expressed in the Δ*cpxR* strain grown under either biofilm or planktonic cell cultures when compared to the WT strain. These genes with known function were further analyzed and ascertained to be involved in adhesion, O-antigen biosynthesis and virulence. In addition, genes involved in invasion were significantly up-regulated in the Δ*cpxR* strain compared to the WT strain. Changes from the planktonic to biofilm state in the WT showed an increased expression of genes involved in chemotaxis, lipopolysaccharide biosynthesis and motility. However, transition from Δ*cpxR* planktonic to Δ*cpxR* biofilm showed a decreased expression of genes involved in chemotaxis and pathogenesis. Further, comparison between WT and Δ*cpxR* strains in biofilms revealed a down-regulation of genes involved in cell adhesion and pilus formation in the Δ*cpxR* strain; however, there was an up-regulation in the expression of genes involved in pathogenesis and motility. Together, these findings shed new light on how fine-tuning

of the CpxR regulatory system may improve survival of *S.* Enteritidis under different environmental circumstances.

4.2.Introduction

The transition of bacterial lifestyle from a free-living single bacteria to a biofilm is a complex developmental process. It is systematically-coordinated and involves interconnected regulatory systems that controls the expression of specific genes contributing to successful biofilm formation. The biofilm formation process can be broadly-classified into several stages that include: i) attachment to the substratum, ii) alteration from a reversible to more secure (incorrectly-termed as being irreversible) binding to the surface, iii) growth of cells within biofilm, iv) maturation of biofilm architecture (Donlan, 2001), and v) dispersion of bacterial cells from biofilm (Gjermansen *et al.,* 2005). Specific environmental signals, genetic elements and molecular mechanisms are required at every stage for successful biofilm formation by the bacteria. Furthermore, bacterial surface appendages like flagella, pili and fimbriae are often associated as crucial components for biofilm formation (Bogino *et al.,* 2013). However, in addition to these surface components, more essential are the regulatory system that control their production and eventually aids in the transition to a biofilm lifestyle. Chapter 3 has highlighted one such regulatory system, i.e. CpxR in two-component Cpx system, that potentially contributes to different stages of biofilm formation in *S.* Enteritidis. Phenotypically, it was observed that deletion of *cpxR* resulted in decreased biofilm biomass and also an unusual filamentation of bacterial cells within the biofilm.

A contradictory role of Cpx in biofilm formation has been demonstrated where Cpx enhances as well as diminishes the expression of genes known to be important for biofilm formation [see review by Raivio (2014)]. Studies have highlighted the involvement of Cpx system that regulates some of the genes/pathways essential in biofilm formation, which includes modulation of specific genes involved in flagellar motility (Price & Raivio, 2009; De Wulf *et al.,* 2002). The transition from the reversible to "irreversible" attachment on the surface is a crucial step in biofilm formation. For similar reasons, it is assumed that the inhibition of motility might promote the biofilm formation (Guttenplan & Kearns, 2013). Cpx response is involved in negatively-regulating the genes involved in motility and chemotaxis. A study on *E. coli* identified target operons *motABcheAW* and *tsr* (involved and motility and chemotaxis) which were down-regulated by activation of Cpx response (Wulf *et al.,*1999). In addition, CpxR is also known to

indirectly diminish the expression of *flhDC* operon (master flagellar regulatory complex) by down-regulating the expression of *flhC* gene (Raivio *et al.,* 2013). There are some studies that suggest that Cpx may hinder biofilm formation. CsgD, a master regulator of biofilm formation (Ogasawara *et al.,* 2011) which activates the curli fimbriae synthesis and extracellular polysaccharides (both considered as features enhancing biofilm formation) is negatively-regulated by activation of the Cpx system. Furthermore, the Cpx system positively-regulates the expression of RprA, a sRNA that inhibits the CsgD expression (Vogt *et al.,* 2014).

Overall, there is currently limited evidence on the precise role of Cpx system in biofilm formation. In fact, only five transcriptomic studies characterizing the Cpx regulon have been published; two in *E. coli*, two in *H. ducreyi* and one recently in *V. cholerae* (Gangaiah *et al.,* 2013; Bury-Moné *et al.,* 2009; Raivio *et al.,* 2013; Labandeira-Rey *et al.,* 2010; Acosta *et al.,* 2015). In this study, I have identified genes that are essential to biofilm formation and its regulation by the Cpx system in *S.* Enteritidis using a transcriptomic approach. I report that activation of Cpx system by deletion of *cpxR*, down-regulates the expression of genes that are known to be essential for biofilm formation, including genes involved in adhesion, O-antigen biosynthesis and virulence. Interestingly, genes involved in invasion were significantly-up-regulation in the Δ*cpxR* mutant strain. I also report a transition in the gene expression between planktonic to biofilm cell condition in both WT and Δ*cpxR* strains. Thus, this chapter reveals previously-unknown details of the transcriptomic profile of *S.* Enteritidis in planktonic and biofilm cell cultures regulated by the Cpx two-component signal transduction system.

4.3.Materials and Methods

4.3.1. Preparation of Planktonic and Biofilm Samples and RNA Extraction

For RNA-sequencing, total RNA was extracted from bacteria grown under planktonic and biofilm condition, as described below. The biomass was collected from the biofilm formed by WT and Δ*cpxR* strains after 48 h of incubation using the method described in section 3.3.6.2. In order to avoid transcriptional changes and RNA degradation, all bacterial samples were prepared in RNA protect bacterial reagent (Qiagen, Valencia, CA, USA) and then stored at -20^{o}C until RNA extraction. For planktonic samples, approximately 10^8 colony forming units (cfu) were pelleted in 1 ml of RNAprotect, incubated at room temperature for 5 min. and pelleted by centrifugation at 1844 g for 10 min. For biofilm samples formed in multi-channel flow cells, the following protocol was used (as described previously) (Korber *et al.*, 1994). Briefly, non-adherent cells were removed by first increasing the flow of the influent medium for 10 min. at laminar flow velocity of 1 cm sec^{-1}. The pump was stopped and the remaining media (10% TSB) from the channels of the flow cell was carefully removed without disturbing the biofilm using 1 cc syringe with sterile needle. A 0.5 ml aliquot of RNAprotect reagent was then injected into the flow cell and the biofilm material was scraped off using a 16-gauge needle. The detached biofilm cells suspended in RNAprotect was then removed using the 16-gauge needle and collected in 1.5 ml micro-centrifuge tubes, followed by centrifugation at 1844 g for 10 min. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and the RNA concentration and purity were quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, USA) and a bioanalyzer (BioAnalyzer RNA 6000 Nano Kit, Agilent Technologies, USA).

4.3.2. RNA-seq Analysis

RNA integrity number (RIN) values of all the samples used for RNA transcriptomic analysis (RNA-Seq) were >6.0. The RNA integrity was also assessed by visualization of the 23S/16S banding pattern using the bleach gel method (Aranda *et al.*, 2012). Library preparation and sequencing were performed at McGill University and Génome Québec Innovation Centre (Montreal, Quebec, Canada) using the KAPA rRNA-depleted (bacteria) stranded library preparation kit (KAPA Biosystems, Wilmington, Massachusetts) and a HiSeq 4000 PE100 sequencer (Illumina, San Diego, CA, USA). All steps were performed according to the manufacturers' protocol, unless otherwise stated. The raw reads obtained from the HiSeq (n=28.1

million paired-end reads) sequencer output were first quality-checked with FastQC_v 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Further, these reads were filtered to remove low-quality sequences in order to eliminate failed reads using Trimmomatic v 0.33 (Bolger *et al.,* 2014). This process included removing (filtering) reads containing poly-N regions, as well as those with low quality. Evaluating sequence quality on the basis of Q30 scores and GC content were also carried out. Filtered reads were mapped to the reference sequence of *Salmonella* serovar Enteritidis P125109 (NCBI GenBank number: AM933172.1 https://www.ncbi.nlm.nih.gov/nuccore/AM933172.1) using Hisat2 v 2.1.0 (Kim *et al.,* 2015). Gene quantification was carried out using cuffquant (Trapnell *et al.,* 2012) and subreads feature counts v1.4.6 (Liao *et al.,* 2013). For differential gene expression, raw read counts were used via an implementation of the edgeR package (Robinson *et al.,* 2009), which employs negative binomial distribution method, in CLC Genomics Workbench (CLCGWB v10.1). Trimmed Mean of *M*-values (TMM) (Robinson *et al.,* 2010) and Upper Quartile (Bullard *et al.,* 2010), both implemented in the edgeR Bioconductor package, were used for normalization of RNA-seq data. Identification of Differentially-Expressed Genes (DEGs) was performed using an Absolute 2-fold difference and Padj [False Discovery Rate (FDR)] <0.05. These steps were carried out at the University of Minnesota Informatics Institute. The list of genes that were significantly expressed where the gene showed > log2-fold change difference (FC) and Padj of <0.05 were filtered and used for further analysis. The Venn diagrams were generated using Venny v 2.1 (Oliveros, 2007). Enriched Gene Ontology (GO) terms from the list of genes regulated by *cpxR*, as well as differentially-expressed genes, were categorized using DAVID (Database for Annotation, Visualization and Integrated Discovery)(Huang *et al.,* 2009) which identifies the major biological pathways. Volcano plots constructed using R and Plot.ly were used to illustrate highly-significant differential gene expression.

4.3.3. Validation of RNA-seq Data using Real-time PCR

Genes that showed significant up- or down-regulation were selected for reverse transcription-PCR (qRT-PCR) to determine the consistency of gene expression data obtained from RNA-Seq on the gene expression. Total RNA was isolated as described above, followed by cDNA synthesis using SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Life Technologies, Carlsbad, CA). Expression differences of genes between WT and Δ*cpxR* strain biofilms were analyzed by real-time PCR using Quantabio Perfecta SYBR Green FastMix (Qiagen, Valencia, CA, USA). The

reaction master mix was prepared as follows: PerfeCTa SYBR Green FastMix (2X) – 10 µl, primers – 1 µl each, RNase free water – 4 µl using 2 µl of template. Total reaction volume – 20 µl. Cycling conditions: 95°C for 30 s, followed by 40 cycles of 95°C – 5 s, 58°C – 15 s, and 72°C – 10 s. Non-template controls, were added to the plate in every qPCR run. Samples were run in triplicate on a Bio-Rad MiniOpticon™ (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). Primer sequences for the genes selected for real-time PCR are listed in Table 4.3.1. DNA gyrase subunit A (*gyrA*) was used as reference control. Results were analyzed using the relative quantification (ΔΔCt) method and expressed as fold change ± SEM.

4.3.4. Experimental Replications and Statistical Analysis

All experimental data represent the arithmetic mean of at least three independent experiments. Biofilm thicknesses were analyzed using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC), and the PROC t-test was used to test for significant ($P < 0.05$) differences.

Table 4. 1. Primers used in Real-Time PCR Gene Expression Assay.

Gene amplified	Primers	Primer sequences (5'-3')	Reference
pegA	pegART-F	GAAGCCCGCACCATTATTAG	(X. Meng *et al*., 2013)
	pegART-R	GAAGTGGTGGGAACATCCTG	(X. Meng *et al*., 2013)
safA	safART-F	GGTTGCTAACACGACACTGG	(X. Meng *et al*., 2013)
	safART-R	CAAAGGTGAACCAGCTCCTC	(X. Meng *et al*., 2013)
hsdS	hsdSRT-F	GGTAACCAACAACTCCCG	(McKelvey, Yang, Jiang, & Zhang, 2014)
	hsdSRT-R	TTGAAAAGACAATCCCACTC	(McKelvey *et al.*, 2014)
dppA	dppART-F	GACATCATCCAGCGGTTTTT	(Doulgeraki *et al.*, 2016)
	dppART-R	TTGACCGTCTGGTCTTCTCC	(Doulgeraki *et al.*, 2016)
gyrA	gyrART-F	GCATGACTTCGTCAGAACCA	(X. Meng *et al.*, 2013)
	gyrART-R	GGTCTATCAGTTGCCGGAAG	(X. Meng *et al.*, 2013)

4.4.Results

4.4.1. Preliminary Comparative Transcriptomic Data Analysis

A significant role of CpxR in biofilm formation was demonstrated in Chapter 3. In this chapter, I further evaluated the genetic differences resulting in decreased biofilm biomass and altered phenotype in the Δ*cpxR* strain. To investigate the underlining genetic differences between the biofilms formed by Δ*cpxR* and WT *S.* Enteritidis strains, a comparative transcriptome analysis was performed, with an overarching goal being to identify the complex transcriptional network controlled by CpxR in *Salmonella* biofilm formation. To achieve this, RNA was extracted from WT and Δ*cpxR* strains grown under planktonic and biofilm conditions [i.e. wild-type biofilm (WB), cpxR biofilm (CB), wild-type planktonic (WP) and cpxR planktonic (CP)] with three biological replicates per treatment, yielding 12 separate transcriptome libraries. The raw reads generated from the 12 libraries were constructed from the above 4 treatments using the Illumina sequencing platform (see Materials and Methods). Overall, an average of 25 million paired end reads were generated per treatment which was subjected to strict quality control criteria to filter the clean reads. Clean RNA reads were mapped to the *S.* Enteritidis P125109 reference genome (NCBI GenBank number: AM933172.1) resulting in >90% of uniquely-aligned sequences for each sample (Table 4.4.1). Differential gene expression was performed using the edge R package with the criteria of >[2x] fold change (FC) difference and adjusted *p*-value less than 0.05.

4.4.2. Differential Expression Patterns of WT and Δ*cpxR S*. Enteritidis Cells Grown Under Planktonic And Biofilm Conditions

In order to obtain a comprehensive overview of the transcriptomic profile between WT and Δ*cpxR* strains under both planktonic and biofilm conditions, four different comparisons were made, i.e. WP vs CP, WP vs WB, CP vs CB and WB vs CB. A total of 4431 genes were expressed; however, only those which were significantly expressed (p-value < 0.05 and FC > [2x]) were qualified for further analysis. In WP vs CP, a total of 832 genes were significantly differentially-expressed, with 394 genes up-regulated and 438 genes down-regulated in the CP treatment. Gene expression differed in WT biofilm (WB) compared to WT planktonic (WP) condition, which showed that a majority of genes were up-regulated under biofilm condition (398 out of 548 genes up-regulated). Comparing the Δ*cpxR* planktonic (CP) vs Δ*cpxR* biofilm (CB), a total 501 genes

were differentially-expressed, with 286 genes were up-regulated and 215 genes down-regulated in Δ*cpxR* biofilm as opposed to Δ*cpxR* under the planktonic condition.

Table 4. 2. RNA-seq data statistics

Samples*	Raw reads	Clean reads	Percentage of mapped reads (%)
WB1	25790067	23421547	98.88
WB2	27757901	25031029	98.73
WB3	30073354	27478006	98.9
CB1	24938984	22652895	92.24
CB2	22855675	21000376	93.86
CB3	31129784	28008190	92.26
WP1	32610868	29687420	98.9
WP2	24782572	22804074	99.05
WP3	22448719	20189183	98.63
CP1	20879098	19163987	91.12
CP2	32661035	29452055	90.85
CP3	41021178	37347978	90.83

*1, 2, and 3 represent independent biological replicates

Under biofilm conditions, 706 genes were significantly differentially-expressed, including 233 genes up-regulated and 473 genes down-regulated in *cpxR* compared to WT biofilms (WB vs CB). The differential genes expressed in all four groups were depicted using a Venn diagram (Figure 4.4.1). From the Venn diagram, it is interesting to note that comparison between WP vs WB revealed 27 genes which were not expressed in the WB treatment; whereas, comparison between CP vs CB treatments revealed 53 genes which were not expressed in CB. There could be two possibilities for the absence of specific genes under biofilm conditions in comparison with planktonic conditions:

i) The genes were not been significantly-expressed under the biofilm condition and hence were not included in the list of significant genes. This does not discount the possibility of their expression under the biofilm condition. Alternatively, the genes were not expressed under biofilm conditions but were expressed under planktonic condition.

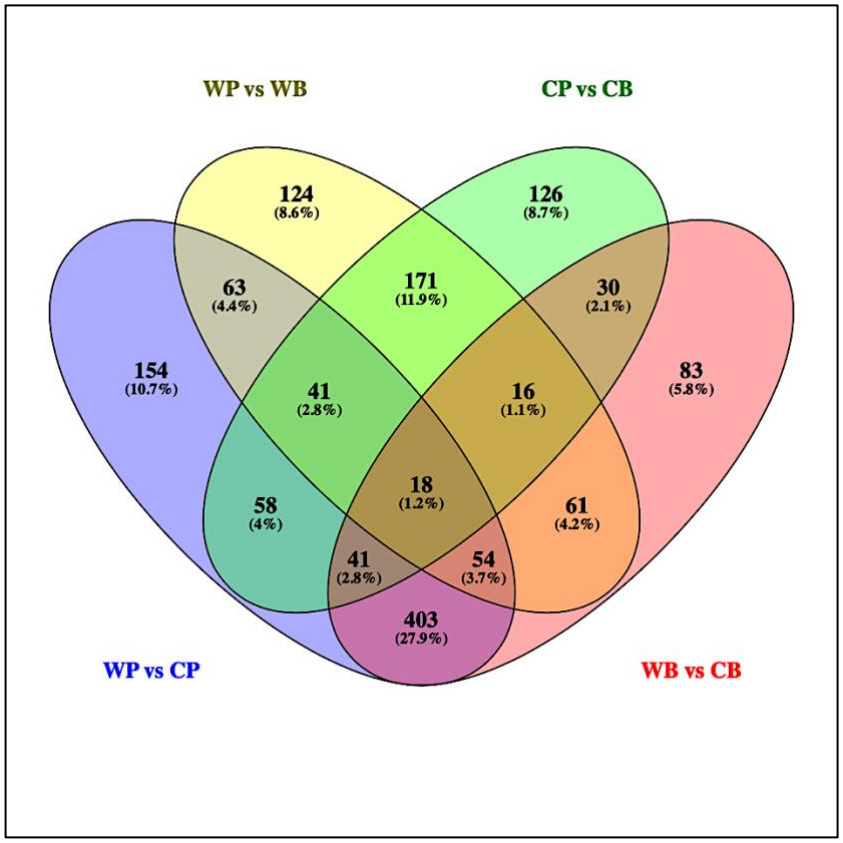


Figure 4. 1. Differentially-expressed genes (DEGs) as depicted using a Venn diagram presentation. All the DEGs were clustered in four comparison groups and are represented by four ellipses. The sum of numbers in one ellipse represent the one comparison group. The DEGs that are common between the groups are represented by the overlapping parts of the different ellipses.

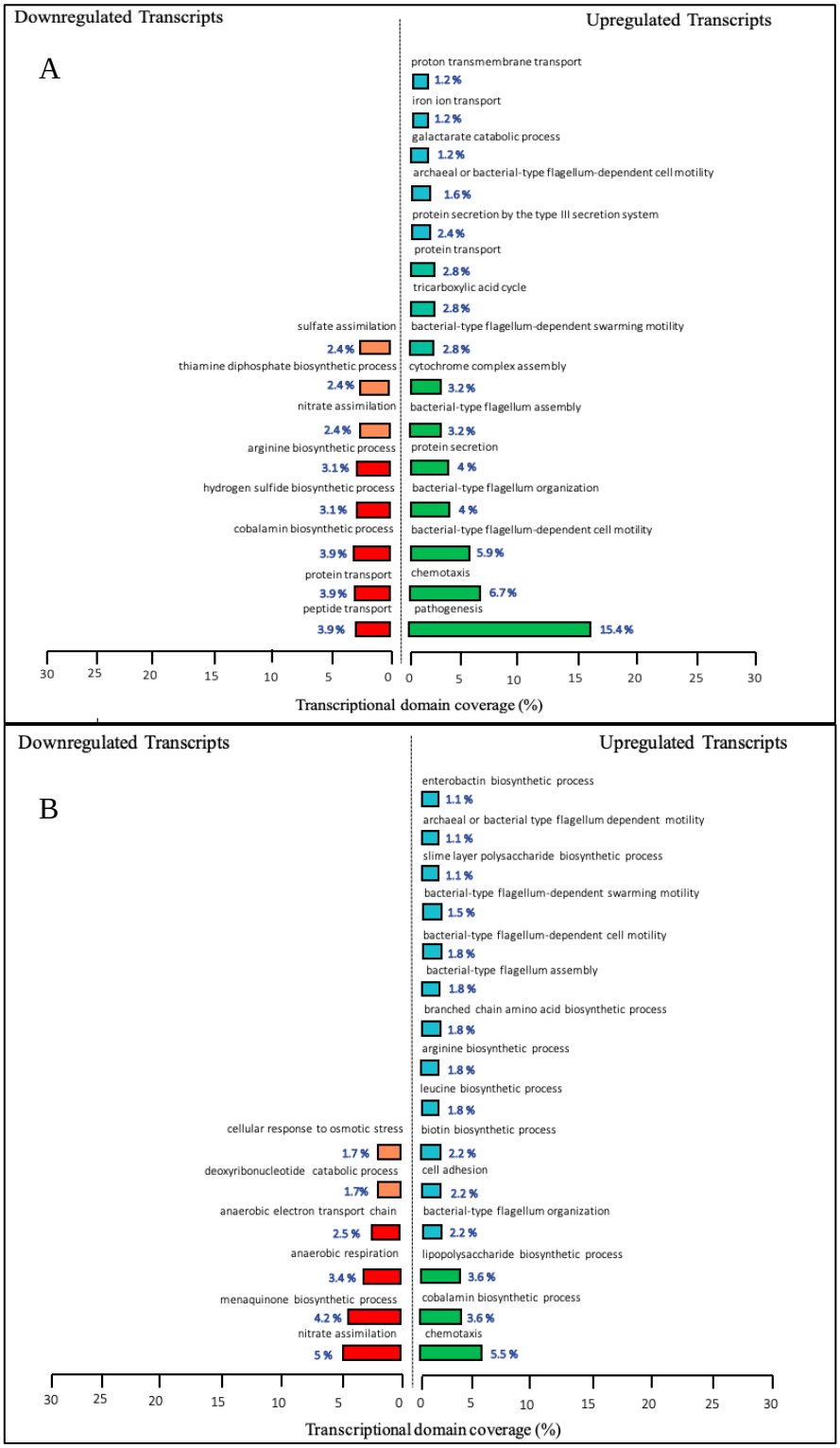
ii) These genes were not essential for biofilm formation and hence their expression was not required for S. Enteritidis. In the case of the Δ*cpxR* strain, it could be possible that the deletion of *cpxR* might have resulted in unexpressed genes under the biofilm condition.

However, in-depth analysis of each gene expressed under both the condition for WT and Δ*cpxR* strains did not pinpoint any specific genes at play which could be classified as being solely-expressed under either the biofilm or planktonic condition. Conversely, differential gene expression was observed following comparison of planktonic WT and Δ*cpxR* strains with biofilm WT and Δ*cpxR* strains. Moreover, comparison of WT and Δ*cpxR* strains revealed that not all the genes expressed in the WT strain were expressed in Δ*cpxR* strain under both biofilm and planktonic growth conditions. This indicates the presence of specific genes that are tightly-regulated by the CpxR regulatory protein. Overall, comparisons between biofilm and planktonic cell cultures, as well as between WT and Δ*cpxR* strains as shown in the below sections, helped to identify the expression of genes essential to biofilm formation and also assisted in identifying the genes specifically-regulated by CpxR.

4.4.3. Functional Classification of Differentially-Expressed Genes (DEGs)

Initially, I characterized the DEGs on the basis of their biological role in order to identify the pathways that were affected due to: i) the transition of state from the planktonic to biofilm condition, and ii) the deletion of the *cpxR* gene in *S.* Enteritidis. Accordingly, enrichment analysis was performed using DAVID on differentially-expressed genes to help understand the biological functions that were significantly enriched in a specific condition. Genes with known function and which were significantly-expressed were assigned to biological functions, the top 15 roles of which (for up and down-regulated genes) are depicted in Figure 4.4.2. In WP vs CP (Figure 4.4.2-A), genes involved in pathogenesis, chemotaxis and motility were significantly-up-regulated in the Δ*cpxR* strain compared to WT under planktonic conditions; whereas, those involved in protein transport were significantly-down-regulated. The up-regulation of genes involved in flagellar synthesis explains an increase in motility observed by the Δ*cpxR* strain reported in the previous chapter. On comparing the DEGs in CP vs CB (Figure 4.4.2-C), down-regulation of genes involved in pathogenesis, chemotaxis as well as in bacterial-type flagellum-dependent motility was observed

in the Δ*cpxR* strain under biofilm conditions. However, genes encoding lipopolysaccharide biosynthesis were significantly-up-regulated in the CB when compared to the CP condition. Comparison of biological function between WP vs WB (Figure 4.4.2-B) showed an up-regulation of genes involved in chemotaxis, cell adhesion, motility and lipopolysaccharide biosynthesis. As known from the literature, these biological functions are directly or indirectly linked with biofilm formation. Finally, a comparison was made between WB vs CB (Figure 4.4.2-D), an analysis that revealed a significant up-regulation of genes in the Δ*cpxR* strain grown under the biofilm condition involved in pathogenesis, the majority of which played an important role during the cell invasion process. Genes encoding proteins having roles in cell adhesin and pilus formation were significantly down-regulated in the CB. Interestingly, genes involved in cell adhesion were not expressed in the planktonic and biofilm comparison for the Δ*cpxR* strain, a finding that indicates these specific genes were not expressed in the Δ*cpxR* strain but were expressed in the WT under both the planktonic and biofilm condition. Details on these genes, as well as other genes which are not expressed in Δ*cpxR* strain but expressed in WT strain, are provided in Section 4.4.5. Thus, the preliminary analysis of DEGs provided a framework of a set of biological processes that are differentially-expressed within the planktonic and biofilm conditions, as well as ones regulated by CpxR. The above highlighted functions having role in biofilm formation and their fine-tuning seems to play an important role during biofilm formation of *S.* Enteritidis.


Downregulated Transcripts
Upregulated Transcripts
A
proton transmembrane transport
1.2%
iron ion transport
1.2%
galactarate catabolic process
1.2%
archaeal or bacterial-type flagellum-dependent cell motility
1.6%
protein secretion by the type III secretion system
2.4%
protein transport
2.8%
tricarboxylic acid cycle
2.8%
sulfate assimilation
bacterial-type flagellum-dependent swarming motility
2.4%
2.8%
thiamine diphosphate biosynthetic process
cytochrome complex assembly
2.4%
3.2%
nitrate assimilation
bacterial-type flagellum assembly
2.4%
3.2%
arginine biosynthetic process
protein secretion
3.1%
4%
hydrogen sulfide biosynthetic process
bacterial-type flagellum organization
3.1%
4%
cobalamin biosynthetic process
bacterial-type flagellum-dependent cell motility
3.9%
5.9%
protein transport
chemotaxis
3.9%
6.7%
peptide transport
pathogenesis
3.9%
15.4%
30 25 20 15 10 5 0
0 5 10 15 20 25 30
Transcriptional domain coverage (%)
Downregulated Transcripts
Upregulated Transcripts
B
enterobactin biosynthetic process
1.1%
archaeal or bacterial type flagellum dependent motility
1.1%
slime layer polysaccharide biosynthetic process
1.1%
bacterial-type flagellum-dependent swarming motility
1.5%
bacterial-type flagellum-dependent cell motility
1.8%
bacterial-type flagellum assembly
1.8%
branched chain amino acid biosynthetic process
1.8%
arginine biosynthetic process
1.8%
leucine biosynthetic process
1.8%
cellular response to osmotic stress
biotin biosynthetic process
1.7%
2.2%
deoxyribonucleotide catabolic process
cell adhesion
1.7%
2.2%
anaerobic electron transport chain
bacterial-type flagellum organization
2.5%
2.2%
anaerobic respiration
lipopolysaccharide biosynthetic process
3.4%
3.6%
menaquinone biosynthetic process
cobalamin biosynthetic process
4.2%
3.6%
nitrate assimilation
chemotaxis
5%
5.5%
30 25 20 15 10 5 0
0 5 10 15 20 25 30
Transcriptional domain coverage (%)

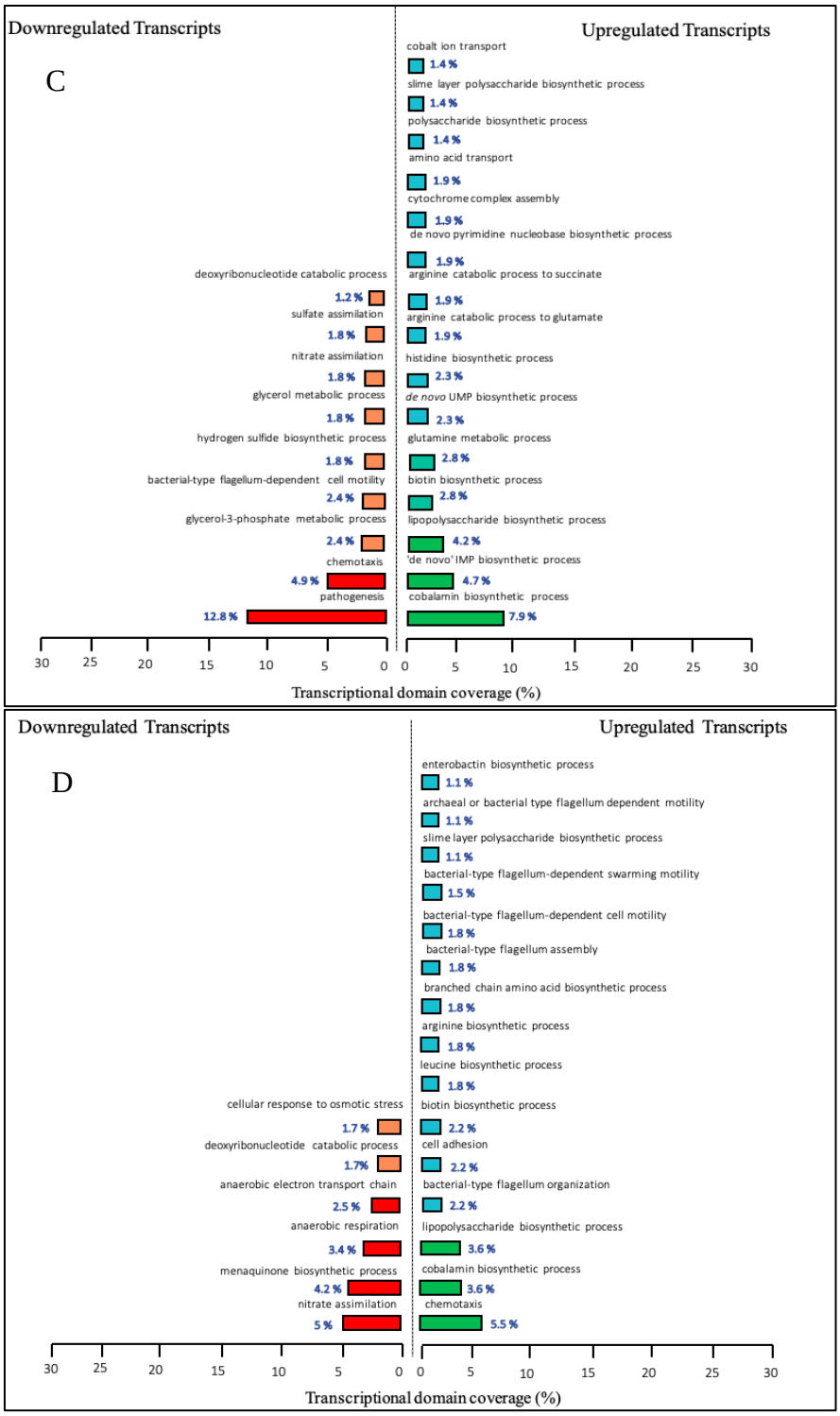


Figure 4. 2. Significantly-enriched gene ontology (GO) terms for differentially-expressed genes in four different groups: (A) enrichment analysis of genes in CP compared to the WP condition, (B) enrichment analysis of genes in WB compared to the WP condition, (C) enrichment analysis of genes in CB compared to the CP condition, and (D) enrichment analysis of genes in CB compared to the WB condition.

4.4.4. Identification of Significantly Up- And Down-regulated Genes In *S.* Enteritidis Biofilm Formed By The Δ*cpxR* Strain

To further investigate the genes that were differentially-expressed in WT and Δ*cpxR* strain biofilms, I focused on the comparison that was made between the WB vs. CB condition. Only those genes with known function and ranging from eight to 25 fold up- or down-regulated (adjusted p value <0.05) out of 706 DEGs were selected for further analysis. DEGs were visualized using a volcano plot presentation, wherein 30 genes were labelled which exhibited significant up- or down-regulation in biofilms formed by the Δ*cpxR* mutant strain when compared to the WT biofilm (Figure 4.4.3). Interestingly, 14 out of 15 genes which were up-regulated had a role in pathogenicity, with most belonging to the Type III Secretion System (TTSS). The TTSS is known to be a major virulence factor in *Salmonella* (Notti & Stebbins, 2016). Based on my analyses, the genes responsible for invasion (*inv*) were significantly up-regulated, which suggests an inhibitory role of CpxR in the regulation of TTSS genes. A study carried out in *S.* Typhimurium showed a significant, but slight, increase in the invasiveness of both *cpxR* and *cpxA* mutant strains; however, the *cpx* mutation did affect the ability of *S.* Typhimurium to infect mice (Humphreys *et al.,* 2004). Thus, the authors concluded that although cell entry was not affected by the Cpx system in *S.* Typhimurium, it has a role in adhesion to eukaryotic cells. A study in *Y. pseudotuberculosi*s showed that a Δ*cpxR* strain invaded epithelial cells more efficiently than WT cells, possibly due to the increased expression of the key invasion factor, Inv (Carlsson *et al.,* 2007). *In vivo* studies investigating the role of CpxR in pathogenesis will provide a better insight in understanding the link between CpxR and pathogenicity in *S.* Enteritidis.

In addition, genes that were down-regulated in Δ*cpxR* biofilm included those having a role in adhesion and ATP-binding protein. Among them there were *pegA* (putative fimbrial subunit protein), *csgB* (nucleation component of curlin monomers) and *fljB* (flagellin). The *csgB* gene, encoding the minor subunit of curli filaments, has a role in aggregation in *E. coli* (Shu *et al.,* 2012). *fljB* plays a role in flagellar phase variation in *Salmonella enterica* (Aldridge *et al.,* 2006). The above genes putatively play a role in biofilm formation and would normally be positively-regulated by the CpxR regulatory protein. Table 4.4.2 (WP vs CP and WB vs CB) and 4.4.3 (WP vs WB and CP vs CB) lists the above 30 genes, along with their fold-changes observed, when compared within all the four treatment groups (WP vs CP; WB vs CB; WP vs WB and CP vs CB).

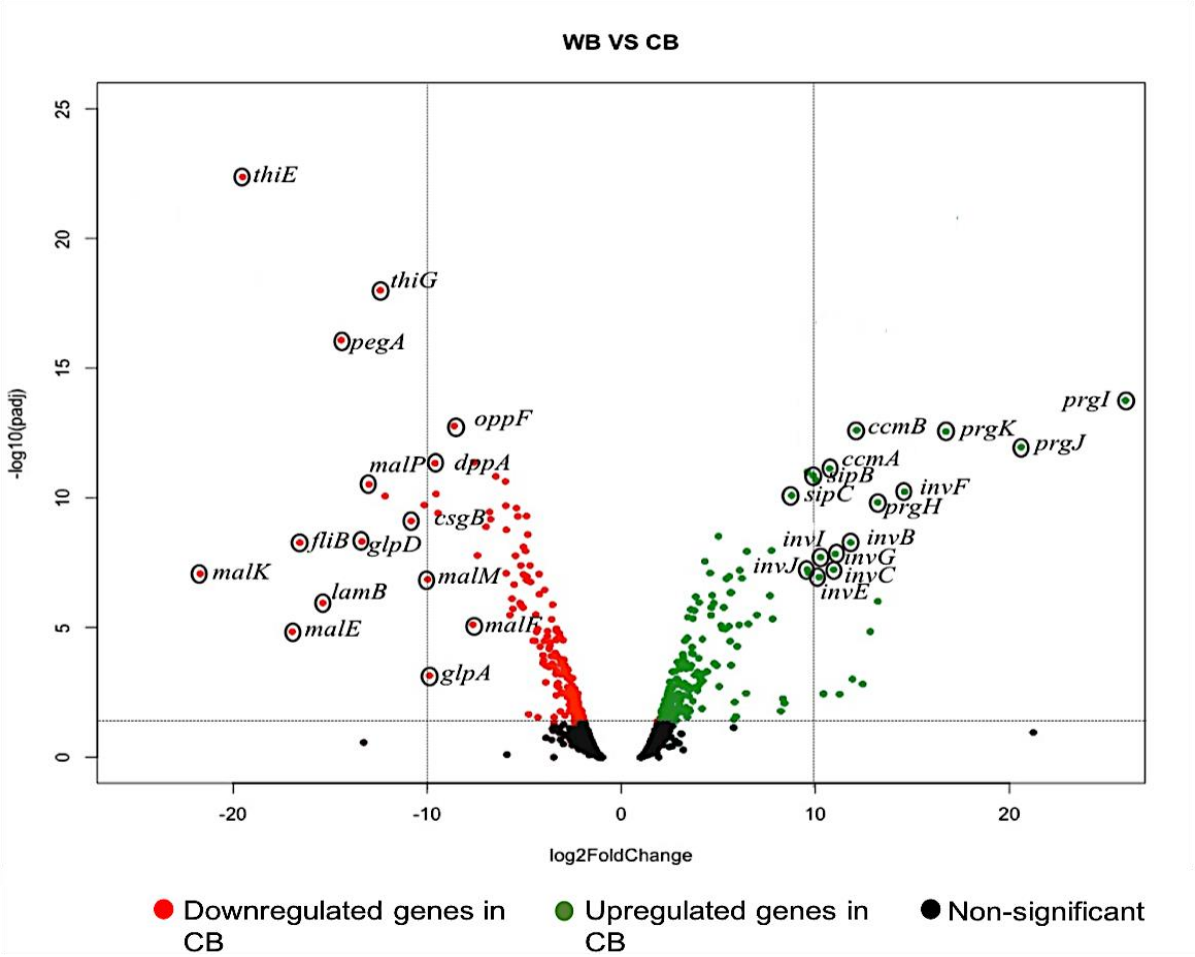


Figure 4. 3. Volcano plot displaying significantly up-regulated and down-regulated genes in the Δ*cpxR* strain grown in biofilms (CB) compared to WT biofilms (WB). The *x-axis* represents the log2 fold-change value and the *y-axis* is the -log10 of adjusted p-value. Selected genes which are eight- to twenty-five-fold differentially-expressed are labelled on the plot

Table 4. 3. List of genes and their fold changes in WP vs CP and WB vs CB

Sr. No	Feature ID	Genes	Gene product	Go Ontology	Log2 fold change in planktonic (WP vs CP)	Log2 fold change in biofilm (WB vs CB)
1.	SEN3949	*thiE*	thiamine-phosphate pyrophosphorylase	thiamine biosynthetic process	-21.0201	-19.504
2.	SEN3946	*thiG*	thiamine biosynthesis protein	thiamine biosynthetic process	-11.3667	-10.761
3.	SEN2145B	*pegA*	putative fimbrial subunit protein		-7.201	-14.430
4.	SEN1293	*oppF*	oligopeptide transport ATP-binding protein	protein transport	-8.239	-8.615
5.	SEN3340	*malP*	maltodextrin phosphorylase		-35.104	-12.169
6.	SEN3454	*dppA*	Periplasmic dipeptide transport protein precursor	Transmembrane transport	-14.094	-9.5996
7.	SEN1905	*csgB*	nucleation component of curlin monomers	cell adhesion	1.0485	-10.825
8.	SEN1049	*fljB*	flagellin	methyltransferase activity	1.0959	-16.56
9.	SEN3351	*glpD*	aerobic glycerol-3-phosphate dehydrogenase	glycerol-3-phosphate metabolic process,	-2.26	-13.35
10.	SEN2266	*glpA*	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	glycerol-3-phosphate metabolic process	-1.456	-9.884
11.	SEN3998	*malK*	maltose/maltodextrin transport ATP-binding protein	ATP-binding cassette (ABC) transporter complex	-69.700	-21.693
12.	SEN4001	*malM*	maltose operon periplasmic protein	carbohydrate transport	-27.206	-9.972
13.	SEN3995	*malF*	maltose transport inner membrane protein	arbohydrate transport	-22.539	-7.647
14.	SEN3997	*malE*	periplasmic maltose-binding protein	maltose transmembrane transporter activity,	-54.105	-16.954
15.	SEN4000	*lamB*	maltoporin precursor	ion transport, cell outer membrane cellular activity	-51.198	-15.376
16.	SEN2715	*prgI*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	105.721	25.981
17.	SEN2714	*prgJ*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	101.2182	20.606

18.	SEN2713	*prgK*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	64.290	16.725
19.	SEN2716	*prgH*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	36.967	13.219
20.	SEN3635	*ccmA*	heme exporter protein A2	Cytochrome complex assembly	11.838	10.062
21.	SEN3634	*ccmB*	heme exporter protein B1	Cytochrome complex assembly	3.634	12.132
22.	SEN2726	*sipB*	pathogenicity island 1 effector protein	Pathogenesis, TTSS	108.229	9.885
23.	SEN2725	*sipC*	pathogenicity island 1 effector protein	Pathogenesis, invasion protein	94.161	8.782
24.	SEN2740	*invF*	possible AraC-family regulatory protein"	Pathogenesis, invasion protein	39.792	14.602
25.	SEN2736	*invB*	secretory protein (associated with virulence)	Pathogenesis, invasion protein	36.769	11.821
26.	SEN2735	*invC*	secretory apparatus ATP synthase (associated with virulence)	Pathogenesis, (TTSS)	29.077	10.940
27.	SEN2738	*invE*	cell invasion protein	Pathogenesis, (TTSS)	10.269	31.327
28.	SEN2739	*invG*	secretory protein (associated with virulence)	Pathogenesis, (TTSS)	11.027	28.999
29.	SEN2733	*invJ*	surface presentation of antigens protein (associated with type III secretion and virulence)	Pathogenesis, invasion protein	30.159	9.682
30.	SEN2734	*invI*	secretory protein (associated with virulence)	Pathogenesis, invasion protein	31.334	9.592

Table 4. 4. List of genes and their fold changes in WP vs WB and CP vs CB

Sr. No	**Feature ID**	**Genes**	**Gene product**	**Go Ontology**	**Log2 fold change in planktonic (WP vs WB)**	**Log2 fold change in biofilm (CP vs CB)**
1.	SEN3949	*thiE*	thiamine-phosphate pyrophosphorylase	thiamine biosynthetic process	-21.0201	-19.504
2.	SEN3946	*thiG*	thiamine biosynthesis protein	thiamine biosynthetic process	-11.3667	-10.761
3.	SEN2145B	*pegA*	putative fimbrial subunit protein		-0.686	-3.79
4.	SEN1293	*oppF*	oligopeptide transport ATP-binding protein	protein transport	-8.239	-8.615
5.	SEN3340	*malP*	maltodextrin phosphorylase		-35.104	-12.169
6.	SEN3454	*dppA*	Periplasmic dipeptide transport protein precursor	Transmembrane transport	-14.094	-9.5996
7.	SEN1905	*csgB*	nucleation component of curlin monomers	cell adhesion	17.41	0.860
8.	SEN1049	*fljB*	flagellin	methyltransferase activity	5.337	-4.932

9.	SEN3351	*glpD*	aerobic glycerol-3-phosphate dehydrogenase	glycerol-3-phosphate metabolic process,	-2.26	-13.35
10.	SEN2266	*glpA*	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	glycerol-3-phosphate metabolic process	-1.456	-9.884
11.	SEN3998	*malK*	maltose/maltodextrin transport ATP-binding protein	ATP-binding cassette (ABC) transporter complex	-69.700	-21.693
12.	SEN4001	*malM*	maltose operon periplasmic protein	carbohydrate transport	-27.206	-9.972
13.	SEN3995	*malF*	maltose transport inner membrane protein	arbohydrate transport	-22.539	-7.647
14.	SEN3997	*malE*	periplasmic maltose-binding protein	maltose transmembrane transporter activity,	-54.105	-16.954
15.	SEN4000	*lamB*	maltoporin precursor	ion transport, cell outer membrane cellular activity	-51.198	-15.376
16.	SEN2715	*prgI*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	1.21	-4.483
17.	SEN2714	*prgJ*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	1.55	-4.26
18.	SEN2713	*prgK*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	1.337	-3.88
19.	SEN2716	*prgH*	pathogenicity 1 island effector protein	pathogenesis, (TTSS)	1.38	-2.80
20.	SEN3635	*ccmA*	heme exporter protein A2	Cytochrome complex assembly	11.838	10.062
21.	SEN3634	*ccmB*	heme exporter protein B1	Cytochrome complex assembly	3.634	12.132
22.	SEN2726	*sipB*	pathogenicity island 1 effector protein	Pathogenesis, TTSS	1.959	-7.55
23.	SEN2725	*sipC*	pathogenicity island 1 effector protein	Pathogenesis, invasion protein	1.56	-9.326
24.	SEN2740	*invF*	possible AraC-family regulatory protein"	Pathogenesis, invasion protein	1.829	-2.06
25.	SEN2736	*invB*	secretory protein (associated with virulence)	Pathogenesis, invasion protein	1.484	-2.966
26.	SEN2735	*invC*	secretory apparatus ATP synthase (associated with virulence)	Pathogenesis, (TTSS)	1.874	-2.033
27.	SEN2738	*invE*	cell invasion protein	Pathogenesis, (TTSS)	1.99	-2.144
28.	SEN2739	*invG*	secretory protein (associated with virulence)	Pathogenesis, (TTSS)	1.906	-1.906
29.	SEN2733	*invJ*	surface presentation of antigens protein (associated with type III secretion and virulence)	Pathogenesis, invasion protein	2.154	-2.045
30.	SEN2734	*invI*	secretory protein (associated with virulence)	Pathogenesis, invasion protein	2.504	-1.819

4.4.5. Identification of Signature Genes Regulated by *cpxR* Involved in Biofilm Formation and Pathogenesis

The Venn diagram suggested the presence of specific genes, which were not expressed by the Δ*cpxR* under both planktonic and biofilm conditions, most relevant transcriptional genes were extracted, and their functions were reported to elucidate the role of *cpxR* in biofilm formation (Table 4.4.4). A total of 223 genes were identified which had little, or no, expression in the Δ*cpxR* strain under either planktonic and biofilm conditions. Among these, ~38% of the genes were assigned to uncharacterized proteins or pseudogenes. A systematic categorization of CpxR-regulated genes/operons affecting biofilm formation are highlighted in the below text.

4.4.5.1. CpxR-Regulated Genes Responsible for Decreased Adherence

Attachment is the first and critical step in biofilm formation and is facilitated by proteinaceous filaments like pili and fimbriae (Koczan *et al.,* 2011; López e*t al.,* 2010; Chagnot *et al.,* 2013). In *S.* Enteritidis, there are 13 fimbrial clusters; my research identified two fimbrial operons, the *saf* operon and the *peg* operon, both of which are directly regulated by CpxR regulatory protein (Thomson *et al.,* 2008). The *Saf* operon is encoded on SPI-6 and includes four genes (*safA, safB, safC* and *safD*) which together encode the major pili subunit, the periplasmic chaperon, the outer membrane usher and the minor subunit, respectively (Zeng *et al.,* 2017). The role of Saf-encoded pili is crucial in biofilm formation and has been highlighted in different organisms, including *Escherichia coli, Streptococcus parasanguis* and *Haemophilus influenzae* (Garnett *et al.,* 2012; Heras *et al.,* 2014; Meng *et al.,* 2011). Furthermore, SafD is highly-conserved in *Salmonella* and has been shown to present a potential vaccination target in mouse model studies (Strindelius *et al.,* 2004).

In contrast to the *saf* operon, the *peg* operon is unique to *S. Gallinarium 287/91, S. Enteritidis and S. Paratyphi A* (Thomson *et al.,* 2008). The *peg* operon consists of *pegA, pegB, pegC and pegD* which respectively encode the fimbrial subunit, fimbrial assembly chaperone, membrane usher proteins and fimbrial protein. *pegB, pegC and pegD* were not found to be expressed in the Δ*cpxR* strain; however, *pegA* was down-regulated in both the planktonic and biofilm condition by $\log_2$ fold change of -7.20 and -14.43, respectively. The role of the *peg* fimbriae

Table 4. 5. Genes Directly Regulated By CpxR Regulatory Protein.

Gene category	Genes or operon	Gene product	Functions reported*	References
Bacterial appendages	*safA* (SPI 6)	major pilus subunit	Proposed role in bacterial aggregation, colonization and biofilm formation. SafD, also exhibited a potential target for vaccination in mice model	(Zeng *et al.*, 2017)
	safB	fimbrial assembly chaperone		
	safC	fimbrial usher		
	safD	fimbrial subunit		
	pegB	fimbrial assembly chaperone	Potential role in oviduct colonization and systemic infection	(Raspoet *et al.*, 2014; Shah *et al.*, 2012)
	pegC	fimbrial biogenesis outer membrane usher protein		
	pegD	fimbrial protein		
	stfF	fimbrial minor subunit	Involved in fimbriae in *S.* Gallinarum	(Felten *et al.*, 2017)
	stfG	fimbrial protein	Involved in fimbriae in *S.* Gallinarum	
	SEN4247	fimbrial protein	Fimbrial operon	(Thomson *et al.*, 2008)
	SEN1976	pilus assembly protein PilV	Predicted to play role during host cell invasion process	(Silva *et al.*, 2012)
	SEN4250 (*sefD*)	fimbrial protein	Potential role of SefD is essential for efficient uptake or survival of *S.* Enteritidis in mammalian macrophage	(Morales, Guard *et al.*, 2012)
	fliD	flagellar hook associated protein	Potential role in filament formation	(Ikeda *et al.*, 1993)
O-antigen biosynthesis	***rfb operon***			
	rfbV	O-chain glycosyltransferase	Encodes enzyme essential for biosynthesis of O-antigen of the LPS.	(Shah *et al.*, 2012)
	rfbX	O-antigen transporter		
	rfbE	CDP-tyvelose-2-epimerase		
	rfbS	CDP-paratose synthase		
	gtr operon			
	gtrA (SPI-17)	bactoprenol-linked glucosyltranslocase	Involvement in epigenetic phase variation; Role in protection against phage killing	(Davies *et al.*, 2013)
	gtrB	bactoprenol glucosyltransferase		
	gtrC	putative lipopolysaccharide modification acyltransferase		
Virulence	***hsd operon***			
	hsdR	type I restriction endonuclease subunit R	Role in protection of the host bacterium from foreign DNA invasion	(Fisunov *et al.*, 2017)
	hsdS	type I restriction-modification protein specificity subunit		
	hsdM	DNA methyltransferase		
	SEN4290	Components of type 1 restriction-modification system	Potential role in pathogenicity	

	SEN429	Components of type 1 restriction-modification system		(Thomson *et al.*, 2008)
	SEN4292	Components of type 1 restriction-modification system		
	SEN1001	Part of type VI secretion system		(Blondel *et al.*, 2009)
	SEN1002	Part of type VI secretion system		

has been established in egg contamination, colonization of chicken, and oviduct including the role of *pegD* during invasion of differentiated Caco-2 cells (Thomson *et al.*, 2008). I further confirmed the expression of *safA* and *pegA* in addition to *dppA* and *hsdS* by using quantitative PCR (qPCR) with the help of the primers listed in Table 3 against the internal reference gene *gyrA*. The expression of these genes was similar to the expression of genes from RNA-Seq, thus confirming our transcriptomic data analysis. In addition to these genes, *stfF* (fimbrial minor subunit), *stfG* (fimbrial protein), *fliD* (flagellar hook associated protein), *SEN4247* (fimbrial protein), SEN4250 (fimbrial proteins) and *SEN1976* (pilus assembly protein PiIV) were also negatively-regulated by CpxR. *SEN1976* gene is linked to biofilm formation and also predicted to play role during host cell invasion process (Silva *et al.*, 2012). SEN4250 (*sefD*) may have role in survival of *S.* Enteritidis against mammalian macrophages by binding to a receptor on the macrophage surface and altering its uptake into the phagocyte for survival under stressful environment (Edwards *et al.*, 2000).

4.4.5.2.CpxR-Regulated Genes Responsible for O-Antigen Biosynthesis

A second cluster of genes directly regulated by *cpxR* are involved in O-antigen biosynthesis. The O-antigen is comprised of repeating oligosaccharide units as a major component of the lipopolysaccharide (LPS) layer, which contributes to bacterial resistance against host immune defenses (Maldonado *et al.*, 2016). A previous study showed decreased biofilm formation in *Actinobacillus pleuropneumoniae* as a result of the absence of O-antigen, thus highlighting the role of O-antigen in biofilm formation in that organism (Hathroubi *et al.*, 2016). In *E. coli*, interrupting the O-antigen biosynthesis induced cell deformation, altering the peptidoglycan (PG) synthesis by sequestering undecaprenyl phosphate (Und-P), a universal lipid carrier (Jorgenson & Young, 2016). Our screening revealed genes that comprised part of the *rfb* and *gtr* operons involved in O-antigen biosynthesis which were not expressed in the Δ*cpxR* strain under biofilm or planktonic conditions. These included *rfbV* (O-chain glycosyltransferase), *rfbX* (O-antigen transporter), *rfbS* (CDP-paratose synthase), *rfbE* (CDP-tyvelose-2-epimerase), *gtrA* (bactoprenol-linked glucosyltranslocase), *gtrB* (bactoprenol glucosyltransferase) and *gtrC* (lipopolysaccharide modification acyltransferase). The above genes are essential for O-antigen biosynthesis and were not expressed in the Δ*cpxR* biofilm, possibly affecting O-antigen biosynthesis and altering PG synthesis, a major structural component of the bacterial cell (Hannaby, 2009). This offers a possible explanation for the unusual cell elongation observed in Δ*cpxR* strain biofilms when visualized

under CLSM. The exact role of the *rfb* and *gtr* genes in *S.* Enteritidis are not known; however, knowledge of their roles may lead to a better understanding of the morphological changes observed in Δ*cpxR* mutant biofilms.

4.4.5.3.CpxR Regulated Genes Involved In Virulence

Among the group of genes that were not expressed in the Δ*cpxR* under biofilm or planktonic conditions are the genes involved in virulence. This includes the type 1 restriction/modification system which protects bacteria from foreign DNA (Pleška *et al.*, 2016). The *hsd* operon, encoding three genes *hsdR (*endonuclease), *hsdM* (methyltransferase) and *hsdS* (specificity subunits), were negatively-regulated in the absence of *cpxR*. Various novel genes regulated by CpxR were also identified, with possible role(s) in *S.* Enteritidis pathogenicity. These include components of the type 1 restriction-modification system (*SEN4290, SEN429,* and *SEN4292*) (Thomson *et al.,* 2008), as well as the type VI secretion system (T6SS) carried on the SPI-19 island (*SEN1001* and *SEN1002*) (Blondel *et al.,* 2009). The *SEN1001* gene encodes for a hypothetical protein containing a LysM domain which are known to be involved in peptidoglycan binding in bacteria (Silva *et al.,* 2012). In *S.* Enteritidis, it was reported that mutation in the *SEN1001* gene resulted in defective colonization of internal organs of infected BALB/c mice. Thus, *SEN1001* was reported to play an essential role in pathogenicity of *S. Enteritidis* in BALB/c mice (Silva *et al.,* 2012).

4.5.Discussion

Transcriptomic profiling, as performed in this chapter, provided a platform to identify the most significant mutation-induced changes observed in Δ*cpxR S.* Enteritidis biofilms when compared to the WT strain in association. Previous studies have also sought to characterize the Cpx regulon in *E. coli*, *H. ducreyi* and recently, *V. cholerae*. Microarray analysis of *E. coli*, *H. ducreyi* and *V. cholerae* identified changes in a number of genes; however, it was reported in *E. coli* as well in *H. ducreyi*, that most of the Cpx-regulated genes were not dependent on CpxR for expression (Gangaiah *et al.,* 2013 ; Bury-Moné *et al.,* 2009; Raivio *et al.,* 2013; Labandeira-Rey *et al.,* 2010; Acosta *et al.,* 2015). Conversely, in *V. cholerae*, over-expression of *cpxR* altered the expression of number of genes involved in energy metabolism, protein fate, cell envelope, transport and binding protein as well as iron acquisition, usage and storage (Acosta *et al.,* 2015). It was concluded that the Cpx response in *H. ducreyi* had evolved to perform different functions as compared to its function in *E. coli*.

Deletion of the *cpxR* gene demonstrated direct transcriptional repression of various genes known to play a role in biofilm formation. It resulted in 31% and 26% of unexpressed genes in the Δ*cpxR* strain under the planktonic and biofilm condition from the list of significantly-expressed genes, respectively. Most of these genes had a role in adhesion, O-antigen biosynthesis and virulence. Furthermore, an in-depth analysis of biological function showed an up-regulation of genes involved in motility in the Δ*cpxR* strain under both planktonic and biofilm conditions when compared to WT *S.* Enteritidis. This, along with motility assay results, reflect the negative regulation of genes involved in motility by CpxR. This suggests that CpxR might have control of the "swim-or-stick" switch which involves the inhibition of flagellar synthesis, thus leading to biofilm formation. Reducing motility by inhibiting the genes involved in flagellar synthesis by CpxR might provide more time for the bacteria to sense and attach to the surface for biofilm formation. Motility is needed to sense/find surfaces, and then once attached, expression of flagellar genes/motility become down-regulated as part of the planktonic to sessile phase conversion). It was also worth noting that the genes involved in adhesion, especially the *pef* and *saf* operons, were not expressed in the Δ*cpxR* strain. Could it be that the expression of motility and adhesion genes are inversely related to each other and CpxR might act as putative checkpoint in the swim-stick phase of biofilm formation? Gaining a better understanding of swim-stick barrier in biofilm

formation can help formulate a more detailed understanding of the role of CpxR during biofilm formation.

As noted in the previous study, the Δ*cpxR* strain showed phenotypic cellular defects, with cells unusually elongated during growth in biofilm. Combing the phenotypic effect to understand the genetic changes behind it, led to several assumptions with respect to phenotypic shifts. The genes involved in O-antigen synthesis were repressed due to the deletion of CpxR. Presumably, this resulted in incomplete production of O-antigen. As described by Jorgenson & Young, the interruption in O-antigen synthesis might have an effect on peptidoglycan synthesis, as both these components require Und-P for synthesizing various glycan polymers (Jorgenson & Young, 2016). Incomplete O-antigen synthesis would result in accumulation of dead-end Und-PP, thus sequestering Und-P for its usage in peptidoglycan synthesis (Jorgenson & Young, 2016). It was also noted in the previous chapter that these observed phenotypic (cell elongation) changes were unstable, and after 72 h cells reverted to the normal (shorter rods) cell phenotype. This could possibly be explained by the occurrence of a suppression mutation which could have prevented the formation of O-antigen intermediates and thus made Und-P available for peptidoglycan synthesis. Interestingly, a study examining *Actinobacillus pleuropneumoniae* demonstrated a decrease in biofilm formation in the absence of O-antigen. The expression of *cpxR* was also significantly reduced (Hathroubi & Hancock, 2015). Further investigation of the *rfb* and *gtr* operons in the absence of *cpxR* might reveal some interesting interactions of CpxR with O-antigen biosynthesis.

Finally, transcriptomic data analysis also shed some light on pathogenesis, revealing some interesting changes in the genome in the absence of *cpxR*. I was able to identify a set of genes involved in virulence that was significantly down-regulated in the Δ*cpxR* strain, including the restriction modification system encoded by *hsd* operon. Several other virulent genes were identified that are known to be involved in virulence, but were not expressed in Δ*cpxR* strain. On the other hand, the genes involved in invasion were significantly up-regulated in the Δ*cpxR* strain compared to WT *S*. Enteritidis. An *in vivo* study in an animal model system could be particularly helpful in subsequent investigation into the role of CpxR in pathogenesis. Nonetheless, augmented invasion and decreased virulence could potentially trigger an immune response by the host which could lead to the development of potential immunity against WT *S*. Enteritidis. A more complete understanding of the CpxR regulator could lead to some practical applications, including the design

of candidate vaccines, in addition to contributing to our broader understanding of the role of CpxR in bacterial stress adaptation.

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5. GENERAL DISCUSSION

At this point, it is worth emphasizing that although extensive research has already been carried out on *Salmonella* [In last ten years (2009-2019), there have been 464,000 papers published on *Salmonella*], it continues to cause a global strain on public health. This is mainly due to its genetic make-up (survival, infection and invasion factors) that permit *Salmonella* to adapt to various challenging environments. Digging deeper into the genetic circuitry of these bacteria could assist in distinguishing the ESR systems that senses and responds to these stressors by modulating the expression of genes, thus providing a protective response or solution to these organisms. Two of these ESR systems that have been extensively studied in my thesis includes the RpoE sigma factor and the CpxR two-component system. Based on previous knowledge and the hypothetical genes regulated by these systems, I primarily focused on the role of these systems during biofilm formation in *S*. Enteritidis.

The first study, as presented in Chapter 3, helped to draw several important conclusions that served as a platform for the second study (Chapter 4) of my thesis. In Chapter 3, I successfully deleted the *rpoE, cpxR* and both *rpoE/cpxR* genes in *S*. Enteritidis, thereby allowing me to, individually and in combination, identify the role that these systems play during biofilm formation. Deletion of *rpoE* resulted in impaired growth; however, no significant differences in auto-aggregation and motility were observed when compared to WT *S*. Enteritidis. Also, biofilm forming ability was not compromised in the absence of *rpoE*. However, deletion of both *rpoE* and *cpxR* severely affected growth rate and significantly reduced motility as well as auto-aggregation ability of *S*. Enteritidis. In addition, biofilm formation was drastically affected, resulting in little or no biofilm biomass being formed over the growth period. Interestingly, the findings reported due to the deletion of *cpxR* were fascinating and offered a logical point of entry into my second study. It was noted that *cpxR* deletion did not impair the growth of the *S*. Enteritidis; however, motility was significantly increased while the auto-aggregation ability was significantly reduced. Further, architectural/morphological characterization of the Δ*cpxR* strain biofilms revealed the presence of a disproportionate population of elongated bacterial cells, many of which exceeded ~50 µm in

length. These elongated/filamentous cells populated the entire flow cell and marked a major departure from the typical architecture observed in benchmark WT *Salmonella* biofilms.

These observations led me to further investigate the genetic factors controlled by the CpxR response regulator. In my second study, using a transcriptomic approach, I characterized the Cpx regulon/transcriptome in the absence of CpxR. Deletion of *cpxR* under both planktonic and biofilm conditions identified several genes which were differentially-expressed, along with a set of genes that were not expressed at all. These unexpressed genes were under direct regulatory-control of CpxR, and identified to be involved in adhesion, O-antigen biosynthesis and virulence. The unusual elongated cell phenotype exhibited by the Δ*cpxR* strain was speculated to be due to the non-expression of genes in *rfb* and *gtr* operons essential for O-antigen biosynthesis. I thus hypothesized that deletion of *cpxR* may have interrupted O-antigen biosynthesis, thereby indirectly interrupting peptidoglycan synthesis and causing cell elongation. Surprisingly, the transcriptomic data also revealed the negative regulation of the TTSS invasion genes by CpxR. However, an *in vivo* study may provide add a greater degree of certainty regarding increased invasiveness of the Δ*cpxR* strain and its role in pathogenicity.

Taken together, these studies provide a detailed view of the transcriptome that were directly regulated by CpxR, with potential roles in pathogenesis and biofilm formation in *S.* Enteritidis. The above data strongly indicates that, CpxR, the response regulator of Cpx system, plays an important role in biofilm formation and directly-regulates various genes with putative roles in the initial stages of biofilm formation. Moreover, my data offers new insights into the potential role(s) of CpxR during pathogenesis events. Further studies will be essential to unravel the extent to which these systems coordinate and regulate gene expression during biofilm formation or pathogenesis, and together may lead to the development of new therapeutics thus easing the bacterial stress on human, plant and animal health.

6. FUTURE DIRECTIONS

The purpose of this thesis was to understand the contribution made by the extra-cytoplasmic stress response systems, in particular, the RpoE sigma factor and Cpx two-component systems, during biofilm formation in *S*. Enteritidis. From the first study, I reasoned that CpxR individually and in conjugation with RpoE, played a key role during biofilm formation. Based on my preliminary data, I proceeded to characterize the Cpx regulon in the Δ*cpxR* strain to further investigate the genes that were regulated by CpxR. Interestingly, my transcriptomic data not only identified the genes directly regulated by CpxR, but also provided an explanation for the various phenotypic changes observed by the Δ*cpxR* strain during planktonic and biofilm cell culture. In addition, the genes affected by the deletion of *cpxR* enabled the formulation of several important questions. Answers to these questions will help elucidate the important and extensive events stemming from the genetic circuitry and their defined connections for bacterial protection against various environmental stressors. Some of these questions and potential paths for securing the answers are listed below:

1) What are the genes that are cooperatively regulated by both the RpoE sigma factor, and CpxR of the Cpx system?

   RNA-seq analysis of the biofilm biomass formed by Δ*rpoE*/ Δ*cpxR* strain harvested at specific time-intervals will enable extraction of sufficient RNA for RNA sequencing. Through RNA-seq analysis, the identification of genes that are mutually up or down-regulated by these ESRs may be identified, allowing further investigations into their role(s) in microbial life cycles, including antibiotic resistance, biofilm formation or pathogenesis.

2) What are the gene(s) that are responsible for the deformation (elongation/ filamentation) of bacterial cells in the Δ*cpxR* strain biofilms.

   Potential genes altered by CpxR and responsible for O-antigen synthesis have been identified in my thesis, and are suspected to be responsible for cell elongation in biofilm condition. Systematically “knocking out” these genes regulated by CpxR, followed by

their growth under biofilm conditions, would help to identify the specific genetic factors and their indirect or direct involvement in alteration of cell morphology via CpxR.

3) Can CpxR be a potential vaccine candidate?

   From my transcriptomic data, it was found that genes involved in invasion were significantly up-regulated in the Δ*cpxR* strain under both planktonic and biofilm conditions. The inclusion of *in vivo* studies using an animal model would help identify the role of CpxR during pathogenesis. Comparison of the Δ*cpxR* and WT *S*. Enteritidis strain's survival in the animal model could direct informed vaccine design and development. An attenuated strain of *S*. Enteritidis, generated by deletion of *cpxR*, might result in increased invasiveness of Δ*cpxR* strain, eliciting an elevated immune response which could qualify CpxR as a potential vaccine candidate against *S*. Enteritidis infection.

4) How would *S.* Enteritidis interact with plants in absence of *cpxR*?

   Plants are known to be an alternate host for various *Salmonella*e, and there are several *Salmonella* outbreaks have been reportedly been linked to plants. The first step of infection is adhesion, which has been noted in many *Salmonella* serovars. From the transcriptomic data, genes having their function in adhesion were significantly down-regulated in the Δ*cpxR* strain. Thus, it would be interesting to investigate *Salmonella*-plant interactions using the Δ*cpxR* strain of *S*. Enteritidis to quantify colonization success.

Overall, my work presented in this thesis helps have provided a broad knowledge base along with "dots" of detail; further research to connect these dots in a more meaningful manner will help to more clearly elucidate how bacteria survive under different stressful conditions.